Disrupting FoxP2 Expression Alters Song Variability and Signal Propagation Through a Basal Ganglia Pathway Important for Learned Vocalizations

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School
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ABSTRACT

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Abstract

Mutations of the *FOXP2* gene impair speech and language development in humans and shRNA-mediated suppression of the avian orthologue *FoxP2* disrupts song learning in juvenile zebra finches. How diminished FoxP2 levels affect vocal control and alter the function of neural circuits important to learned vocalizations remains unclear. Using a combination of behavioral analysis, *in vivo* intracellular recordings in anaesthetized birds, pharmacology and extracellular recordings in singing birds, I addressed how FoxP2 knockdown in songbird striatum affects vocal control and signal propagation through circuits important for the control of learned vocalizations. In summary, I found that FoxP2 knockdown in the songbird striatum disrupts developmental and social modulation of song variability. Recordings in anaesthetized birds show that FoxP2 knockdown interferes with D1R-dependent modulation of activity propagation in a corticostriatal pathway important to song variability, an effect that may be partly attributable to reduced D1R and DARPP-32 protein levels. Furthermore, recordings in singing birds reveal that FoxP2 knockdown prevents social modulation of singing-related activity in this pathway. These findings show that reduced FoxP2 levels interfere with the dopaminergic modulation of vocal variability, which may impede song and speech development by disrupting reinforcement learning mechanisms.

Dedication

This thesis is dedicated to the loving memory of my grandmother and grandfather.

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I would like to sincerely thank my advisor Prof. Richard Mooney for his guidance, immense support and relentless encouragement without which this work would be impossible. I am greatly indebted to Rich for providing me with freedom to try different things, while kindly and firmly guiding me in the right direction. I'm certain that Rich will continue be an inspiration for the rest of my life. I would like to thank all the past and present members of the Mooney Lab with whom I have had the pleasure of working. It is never easy being an international student and they made sure that I always felt at home. Whether it was helping out with experiments, conceiving elaborate Rich Rolls, or cracking up over ridiculous YouTube videos, they made work feel like so much fun. A special shout out to Dr. Kosuke Hamaguchi for teaching me how to perform chronic recordings, analyze data and for being extremely generous with his scripts. I would like to thank Stephen Harward, who so willingly helped out with the Western Blot experiments and never complained about all the hours he spent troubleshooting. Thanks to all my committee members for their constructive criticism and suggestions that helped improve this work. I would like to thank my parents for their love, endless support and encouragement. Thanks to all my friends for making life in Durham thoroughly enjoyable. Last but not least, I am grateful to my husband Raj for believing in me, for getting me through the bad days, for keeping me well fed when I got 'hangry' and importantly for always being there for me.

1. Introduction

Speech and language form an integral part of human communication. It is estimated that an average of 7-8% of children suffer from speech and language disorders (Tomblin et al., 1997). Despite the prevalence and debilitating nature of speech and language disorders, the molecular mechanisms and neural circuits that underlie these disorders remain largely unknown. The discovery of mutations in the *FOXP2* gene[1] in a large multi-generational family (KE family), containing several individuals with orofacial dyspraxia (Lai et al., 2001) has provided us with an unique opportunity to better understand the molecular mechanisms and neural circuits that underlie speech and language disorders.

1.1 Mutations of the FOXP2 gene result in verbal dyspraxia.

The discovery of the *FOXP2* gene as a cause of verbal dyspraxia in the KE family has been so far the first and only instance in which mutations of a single gene have been linked to a speech and language disorder with no major insufficiencies in hearing and intelligence. *FOXP2* is a Forkhead box family gene, located at the SPCH1 (speech-and-language-disorder-1) locus of the human chromosome 7, and it encodes a transcription factor (Lai et al., 2001; Fisher et al., 1998; Lai et al., 2000). Humans with *FOXP2* mutations

[1] The human version of the gene is referred to as *FOXP2*, the mouse variant, *Foxp2* and the avian ortholog, *FoxP2*. For the sake of convenience, the term *FoxP2* will be used in instances that discuss more than one species. For more information on FOX gene nomenclature refer to Kaestner et al., 2000.

fail to develop normal speech and language (Hurst et al., 1990; Watkins et al., 2002a; Vargha-Khadem et al., 2005; Feuk et al., 2006; MacDermot et al., 2005). The 'core' deficits in patients with *FOXP2* mutations appear to be an inability to control fine orofacial movements, severe deficits in articulation (Hurst et al., 1990) and trouble with fluent repetition of word and non-word sequences (Watkins et al., 2002a). Interestingly, when the oral praxis (e.g. clicking the tongue, biting the bottom lip etc.) of affected subjects were tested, their performance was poorer compared to the unaffected group (Watkins et al., 2002a). However, limb praxis (e.g. combing hair, demonstrating the use of a key) remained unaltered in patients with *FOXP2* mutations. Furthermore, children with *FoxP2* mutations are unable to laugh and cough spontaneously (Rice et al., 2011). Taken together, these studies point to a role for FOXP2 in motor planning and control.

However, it is important to note that in addition to deficits in speech, human subjects with *FOXP2* mutations exhibit deficits in expressive and receptive language (Hurst et al. 1990; Vargha-Khadem and Passingham 1990; Vargha-Khadem et al. 1995, 1998; Rice et al., 2011). The hearing ability of these patients is unimpaired. Furthermore, affected human subjects have lower performance intelligence quotient and verbal intelligence quotients compared to control individuals (Watkins et al., 2002a). Nevertheless, these lower scores likely undervalue cognitive ability and intelligence, as speech and language deficits in these human subjects introduce severe confounds in the interpretation of these results. In support of this idea, affected and control individuals

have comparable scores in non-verbal IQ subtests (e.g. picture completion, block design, object assembly) (Watkins et al., 2002a, Rice et al., 2011). In summary, while these human studies point towards impaired motor control playing an important role in the speech deficits observed in humans with *FOXP2* mutations, distinguishing the role of FOXP2 in the acute control of vocal motor variability from developmentally restricted speech learning mechanisms remains complicated, in part because *FOXP2* is expressed in humans from embryonic development onwards (Teramitsu et al., 2004). I hypothesize that reduction in functional levels of FOXP2, affects the acute control of vocal variability important to vocal learning. I tested this hypothesis in Chapter 2 of this study using the ability of adult male zebra finches (a vocal learning species) to modulate song variability as function of social context as an assay.

1.2 Neural circuits affected by FOXP2 mutations

Brain imaging studies in the affected members of the KE family provided the first insight into the different regions affected by *FOXP2* mutations. *FOXP2* mutations resulted in lower levels of grey matter in the head of the caudate nucleus (a region of the striatum implicated in sensorimotor integration important to head and face movements), Broca's area and the cerebellum (Vargha-Khadem et al., 1998; Belton et al., 2003), regions of the brain historically implicated in speech and language dysfunction. Furthermore, functional brain imaging studies (fMRI and PET) in the affected members of the KE family have revealed abnormal activation patterns in the Broca's area, caudate nucleus and putamen

during the performance of word repetition tasks (Vargha-Khadem et al., 1998; Liégeois et al., 2003).

1.2.1 Striatal deficits play an important role in the speech and language deficits observed with *FOXP2* mutations

While *FOXP2* mutations affect multiple brain regions, several lines of evidence implicate anomalous striatal function in the speech and language deficits observed in humans with *FOXP2* mutations (Lai et al., 2001; Lai et al., 2003). FOXP2 The first line of evidence comes from imaging studies in humans with *FOXP2* mutations, which reveal a significant reduction in the volume of the head of the caudate nucleus. The reduction in the volume of the caudate nucleus significantly correlated with performance on a test of oral praxis, with smaller volumes corresponding to lower oral praxis scores (Watkins et al., 2002b; Belton et al., 2003). Second, while FOXP2 is expressed in both the striatum and the cerebellum, 'knock in' of the humanized FoxP2 in mouse affects synaptic long-term depression (LTD) in the striatum but not in the cerebellum (Reimers-Kipping et al., 2010). Third, no behavioral phenotype was observed with cortical deletions of FoxP2, although behavioral deficits arise with cerebellar and striatal deletions of the *Foxp2* gene (French et al Abstract# 57.09/J9 SFN 2012). Finally, the most important clue comes from lentiviral shRNA-mediated reduction (i.e., knockdown) of FoxP2 levels in a striatopallidal structure (Area X) alone of juvenile male zebra finches, which resemble humans in their capacity for imitative vocal learning. This spatially restricted knockdown is sufficient to prevent them from accurately copying a tutor song (Haesler et al., 2007).

1.3 Disrupting Foxp2 expression in mice

Foxp2 expression patterns in mice are highly comparable to humans (Lai et al., 2003). Mouse pups that are homozygous for the mutant *Foxp2* allele (R552H mutation corresponding to the KE family) have significant delays in motor development and die within 3 weeks of birth (Shu et al., 2005). Heterozygous Foxp2 knockout (KO) mice have lifespans comparable to the control animals. There is contradictory evidence on whether heterozygotic Foxp2 KO mice have delays in motor development (Shu et al., 2005; French et al., 2007; Groszer et al., 2008). Because of the severe developmental deficits, motor delays and postnatal lethality, the use of homozygotes to study Foxp2 function is limited and therefore the term Foxp2 KO mice for the rest of this thesis will refer to mice heterozygous for Foxp2 mutations unless otherwise noted. Foxp2 KO mice show deficits in motor skill leaning, acquiring the ability to run on an accelerated rotarod or a tilted running wheel more slowly and more poorly compared to control animals (Groszer et al., 2008).

Interestingly, expressing the mutant *Foxp2* gene in the striatum of mice results in shorter dendrites, fewer spines, and abolished LTD in the medium spiny neurons (MSNs) (Groszer et al., 2008), an important correlate of striatum-dependent learning (Gerdeman et al., 2002; Yin et al., 2006). In addition to striatal abnormalities, the Foxp2 KO mice exhibit subtle deficits in paired pulse facilitation in the Purkinje cells of the cerebellum. Conversely, 'knock in' of the humanized Foxp2 results in increased dendritic length and

augmented LTD in the MSNs (Enard et al., 2009; Reimers-Kipping et al., 2010). Recent studies have also shown that mice with Foxp2 mutations are impaired in auditory-motor associative learning (Kurt et al., 2010) and also display patterns of striatal (MSN) activity that are abnormally high and aberrantly modulated during motor skill learning (French et al., 2012).

Mice produce vocalizations in both the audible (~20 Hz–20 kHz) and ultrasonic (> 20 kHz) range (Ehret, 2005). Mice pups will produce ultrasonic isolation calls when separated from their mothers and produce audible and ultrasonic distress calls when handled by humans. Contrary to initial findings (Shu et al., 2005) it appears that FoxP2 KO pups are not affected in their ability to produce ultrasonic isolations and distress calls (Groszer et al., 2008; Gaub et al., 2010). However, the question still remains whether Foxp2 mutations affect ultrasonic 'songs' of adult male mice (Holy and Guo, 2005).

In summary, although *Foxp2* mutations in mice result in numerous neural and behavioral deficits, how these deficits relate to vocal imitation is unclear, in large part because several lines of evidence indicate that the mouse vocal repertoire is innate. First, cross fostering experiments showed that mice sang ultrasonic songs that shared acoustic characteristics with of songs of their genetic parent strain and not the mice strain they were raised with (Kikusui et al., 2011). Second, ultrasonic songs produced by deaf mice (otoferlin-knockout) were comparable to songs produced by their heterozygous siblings and wild-type mice (Hammerschmidt et al., 2012). Finally, an extensive analysis of

ultrasonic songs produced by mice engineered to be chronically deaf by the expression of diphtheria toxin in inner and outer hair cells (causing them to die) produced vocalizations that were remarkably similar to their control counterparts (Mahrt et al., 2013). Taken together, majority the available evidence suggests that ultrasonic vocalizations produced by mice are innate. While mice are not vocal imitators, it appears that in some instances mice are capable of modifying their song pitch in response to social experience (Arriaga et al., 2012).

The behavioral deficits resulting from altered Foxp2 levels mostly involve motor skill learning, a trait that does not appear to be affected in humans with *FOXP2* mutations (Enard et al., 2009; Groszer et al., 2008; Gaub et al., 2010; Watkins et al., 2002a). Therefore, while studies in mice have furthered our understanding of structural and functional changes in striatal neurons that result from *FOXP2* mutations (Enard et al., 2009; Groszer et al., 2008), how these changes affect neural circuits important to learned vocal control remain poorly understood.

1.4 Songbirds as a model system to study disrupted FoxP2 function

Songbirds afford a powerful system in which to understand how alterations in FoxP2 expression affect the function of striatal circuits important to learned vocalizations and to determine whether FoxP2 plays a role in adult vocal motor control in addition to its established role in juvenile vocal learning. Song learning in birds and speech acquisition in humans share many parallels (Doupe and Kuhl, 1999). To highlight a few,

both humans and songbirds have innate predispositions to species-specific vocalizations. Both species learn their vocalizations during early time points in development and rely on auditory feedback to both learn and maintain their vocalizations later in life. Song learning in birds, and speech learning in humans occur during critical periods. Finally, neural circuits for song learning and speech acquisition share many similarities.

1.4.1 Vocal learning in songbirds parallels human speech learning in many ways.

Songbirds belong to small group of animals that have the capacity to learn vocalizations, which also includes humans, bats and cetaceans (Janik and Slater, 1997). Zebra finches have been a popular songbird for studying neural circuits and mechanisms that underlie vocal learning in part because of the ease at which they breed in captivity, the remarkable fidelity with which they copy their tutor songs, the highly quantifiable nature of their song and the well characterized neural circuitry for song learning and production (Figure 1). A juvenile male zebra finch learns the song of an adult male tutor with remarkable precision and the vocal learning process in zebra finches involves two distinct but overlapping phases, a sensory phase followed by a sensorimotor phase (Konishi, 1965; Immelman, 1969; Marler, 1970).

During the sensory phase that lasts between 20-45 days post hatch (dph), the juvenile bird listens to and memorizes the song of his tutor (usually, his father). The sensory learning phase is followed by a more prolonged sensorimotor phase (~45-90 dph), a period of motor exploration when the juvenile bird produces more variable plastic song.

During this period a juvenile zebra finch uses auditory feedback to guide his vocal output to match the memorized tutor song (Marler, 1970). The amount of song variability decreases over the course of development and at the end of the sensorimotor phase (~ 90 dph); coincident with sexual maturity the song becomes highly stereotyped in a process referred to as 'crystallization'.

The crystallized adult male zebra finch song consists of a motif made up of an invariable sequence of syllables separated by gaps and the syllables are characterized by remarkable spectro-temporal precision. In addition to relying on auditory feedback for song learning, some species, including the zebra finch are dependent on auditory feedback to maintain stable adult songs. Deafening or exposure to delayed auditory feedback (DAF) causes the stereotyped song to become more variable and degrade (Konishi 1965, Leonardo and Konishi 1999).

Several lines of evidence suggest that regulation of song variability required for motor exploration is an important ingredient for both song learning and maintenance. Reinforcement models of song learning posit that juvenile birds produce variable song to explore the available vocal motor space and use auditory feedback to reinforce those renditions that best match the memorized tutor song in order to improve its performance (Doya and Sejnowski 2000, Fiete et al., 2007; Fee and Goldberg, 2011). A popular model is that juvenile birds harness song variability to explore a vocal motor space and use auditory feedback to reinforce those renditions that best match the tutor song. While a

direct link between linking acute song variability and song learning remains elusive, it is likely that acute motor exploration will play a critical role in vocal imitation. In support of this idea, as sensorimotor learning progresses the amount of song variability and Wiener entropy (is a measure of noisiness of a syllable with a lower value corresponding to less noisy syllable) produced by juvenile bird decreases (syllables become less noisy; Tchernichovski et al., 2001) and the at end of this sensorimotor learning they begin to sing a highly stereotyped crystallized song. These studies suggest that the ability to regulate motor is essential to song learning. Therefore, deficits in the control of acute variability could in turn affect the fidelity with which birds copy their tutor songs.

1.4.2 Acute control of song variability in adult birds

Male zebra finches use their songs primarily to court female and while the songs they produce are highly stereotyped, they are capable of modulating certain features of their songs as a function of social context. An adult male zebra finch produces songs in two different social contexts, those produced when singing alone (referred to as 'undirected song') and those directed at a female (referred to as 'directed song') (Sossinka and Böhner, 1980; Kao and Brainard, 2006). Remarkably, undirected songs have syllables with greater trial-by-trial variability compared to directed songs (Kao and Brainard, 2006). In addition, the directed songs are faster and each motif contains more introductory elements and there are more motifs per bout compared to the undirected song (Sossinka and Böhner, 1980, Kao and Brainard, 2006). This ability to modulate song variability in the

presence of female birds has immense ethological significance and likely affects the reproductive success of the animal. Both socially naïve and mated females discriminate between directed and undirected songs and strongly preferred the less variable directed songs and this was demonstrated by their willingness to spend more time near speakers that broadcasted directed songs over those that played undirected songs (Woolley and Doupe, 2008).

Furthermore, recent contingent reinforcement experiments have shown that adult songbirds are able to control this residual song variability to alter specific features of their song in order to avoid punishment (usually loud white noise or distorted auditory feedback) (Tumer and Brainard, 2007). In addition, adult songbirds are capable of changing the pitch of individual syllables in order to compensate for perceived errors when the pitch is distorted using custom designed headphones (Sober and Brainard, 2009). In summary, these findings demonstrate that while adult male zebra finches sing highly stereotyped songs, they have residual song variability that they are able to modulate as a function of social context and are able to harness this residual variability to change specific spectral features in order to maintain their song.

In this study (Chapter 2) I use the ability of adult birds to change song variability in a context-dependent manner to assay the effect of reduced FoxP2 levels on the acute control of song variability. Furthermore, use of adult birds allowed us to dissociate the effects of FoxP2 knockdown on ongoing adult motor (vocal) control from effects during

juvenile learning. Finally, adult birds provide a stable baseline for song behavior from which subtle deviations that result from reduced FoxP2 levels can be more easily discerned.

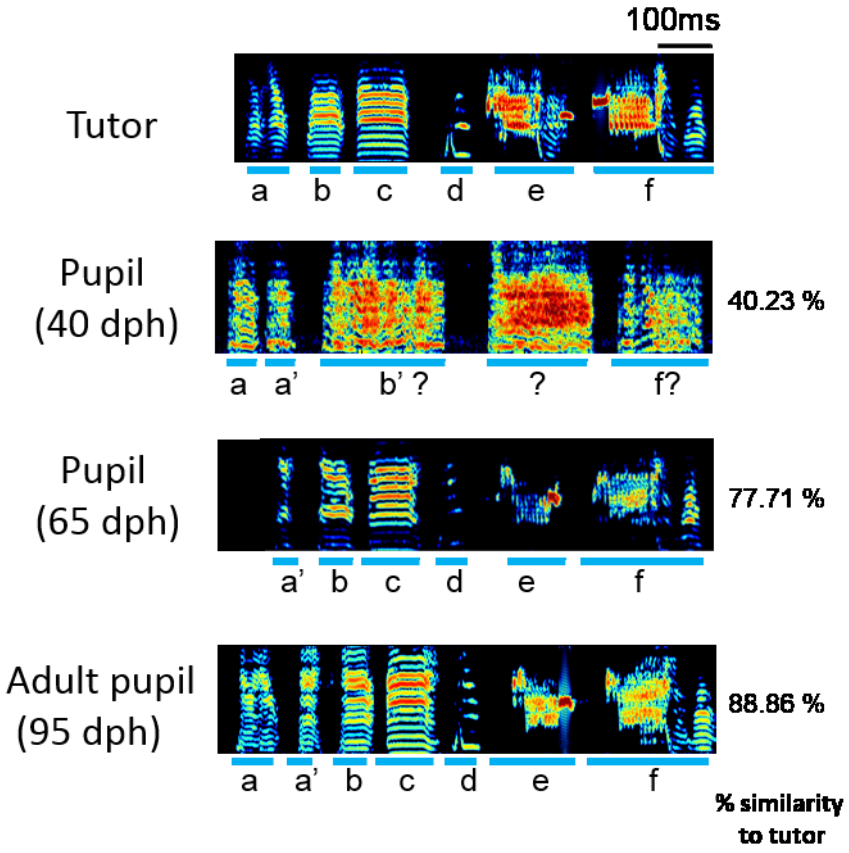

Figure 1: Juvenile zebra finches learn their tutor's song with remarkable precision

Example sonograms of a tutor's song (top) and the songs of a juvenile zebra finch (bottom three, at 40, 65 and 100 dph, days post hatching) who copied from the tutor. The pupil's song becomes more similar to the tutor's song over the course of development. After crystallization the adult pupil sings a stereotyped song that is a close match to the tutor's song (% similarity score at 100 dph is 88.86). Blue lines indicate individual syllables, the scale bar indicates 100ms and the ordinate is 0-9kHz.

1.5 Neural circuits for song learning and production

The songbird brain contains well-characterized song control circuitry (Figure 1A). The song system consists of two distinct but interconnected pathways – the song motor pathway (SMP) and the anterior forebrain pathway (AFP) (Figure 2). The pathways arise from two different projection neuron (PN) classes in the song premotor nucleus HVC (used as a proper name), thought to be analogous to Broca's area in humans. The SMP is required for song production in juveniles and adult birds, it involves a direct projection from one PN neuron type (HVC_{RA} neurons) to the song motor nucleus RA (robust nucleus of arcopallium; analogue of primary motor cortex in humans), which in turn projects to the brainstem nucleus that control the syringeal and respiratory muscles. A popular model is that HVC_{RA} neurons function as a clock and that the activity of these neurons encodes a precise timing signal (Hahnloser et al., 2002). In support of this idea, cooling HVC causes the song to stretch (Long and Fee, 2008). It is believed that the RA neurons encode the acoustic features of individual syllables (Leonardo and Fee). Lesions in the SMP permanently disrupt singing (Nottebohm et al., 1976). Moreover, HVC microstimulation leads to truncation of an ongoing syllable and resetting of the motif (Wang et al., 2008). Furthermore, tutor-song triggered electrical or optogenetic microstimulation of HVC in juvenile birds during exposure to tutor song disrupts song learning (Roberts el al., 2010).

The anterior forebrain pathway (AFP) plays a critical role in song learning and maintenance but is not essential for song production (Nottebohm et al, 1976; Scharff and Nottebohm, 1991). The AFP provides an indirect pathway to connect the HVC (HVC_X projection neurons) to the RA via Area X (striatopallidal structure), DLM (medial nucleus of the dorsolateral thalamus) and LMAN (lateral magnocellular nucleus of the anterior nidopallium; the output nucleus of the AFP). The AFP shares numerous anatomical and functional similarities with the mammalian corticostriatal circuitry. Area X contains striatal medium spiny neurons (MSNs) that share morphological and functional similarities with their mammalian counterparts, and receive gluatamergic input from HVC. Furthermore, like in the mammalian striatum, these neurons receive dense dopaminergic input from midbrain dopaminergic nuclei, the ventral tegmental area (VTA) and substantial niagra (SNc) (Lewis et al., 1981; Bottjer et al., 1989; Bottjer, 1993; Soha et al., 1996). In addition, the output neurons of Area X, the pallidal neurons share many similarities with the mammalian pallidal neurons (Goldberg et al., 2010). Also, important to this study, there is a high level of expression of the FoxP2 protein in the MSNs of Area X (Doupe et al., 2005; Fisher and Scharff, 2009). Therefore, making songbirds an ideal model system to understand the nature of corticostriatal deficits that result from FoxP2 knockdown and how these circuit deficits affect song control.

In addition to being required for song learning, the AFP plays an important role in the acute control of song variability in juvenile and adult birds. Lesions to Area X in

juvenile birds lead to increased song variability and a failure to crystallize, while lesions and reversible inactivation of LMAN significantly decreases in song variability in both juvenile and adult animals (Scharff and Nottebohm, 1991; Olveczky et al., 2011; Kao and Brainard, 2006). Furthermore, LMAN lesions prevent deafening or DAF induced song deterioration, suggesting that the AFP plays an important role in evaluating song performance important for both song learning and song maintenance (Brainard and Doupe, 1999; Andalman and Fee, 2009, Warren et al., 2011). LMAN neurons exhibit higher trial-by-trial variability, increased firing rates and augmented bursting activity in while singing variable undirected songs in adult birds and singing variable plastic song in juveniles (Kao et al., 2005; Olveczky et al. 2011). This trial-by-trial variability and augmented bursting activity in LMAN is modulated by social context and reduced during directed singing. Importantly, song-triggered microstimulation of LMAN induces acute and specific changes in song features (Kao et al., 2005), showing that altering LMAN activity acutely affects song variability. Furthermore, LMAN lesions in adult male zebra finches abolish social context-dependent changes in song variability (Kao and Brainard, 2006).

Thus, these findings demonstrate an important role for LMAN neurons in driving context-dependent song variability in adult birds and song variability important for vocal exploration in juvenile birds and raising the possibility that changes in LMAN activity could underlie the increased song variability observed in FoxP2 knockdown birds

(Chapter 2). I hypothesize that FoxP2 knockdown abolishes context dependent differences in LMAN activity, and I tested this hypothesis using chronic extracellular recording techniques to record the activity of LMAN neurons in singing birds as they switched between social contexts (Chapter 3).

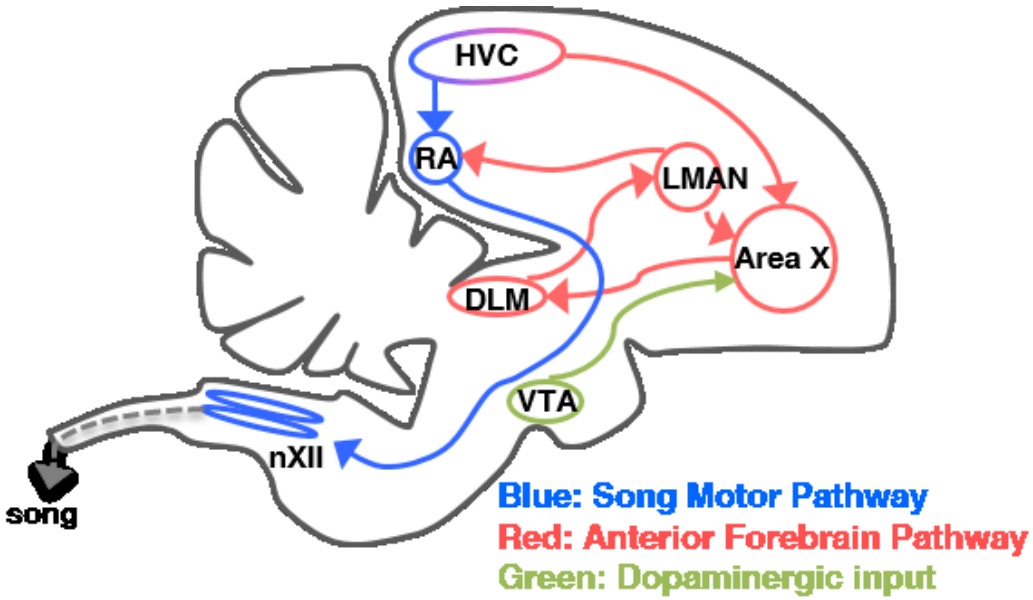

Figure 2: A simplified diagram of the song system in the zebra finch brain

The song motor pathway (shown here in blue) is required for song production and song learning. The anterior forebrain pathway (shown here in red) is required for song learning and the acute control of motor variability. HVC used as a proper name; RA, robust nucleus of the arcopallium; Area X, striatopallidal component of the song system; LMAN, lateral magnocellular nucleus of the anterior nidopallium; DLM, medial nucleus of the dorsolateral thalamus; nXII, nucleus XII. Area X receives dense dopaminergic input from the ventral tegmental area; VTA (green). Some structures and connections are omitted for simplicity.

1.6 FoxP2 expression and its function in songbirds

The FOXP2 protein is highly conserved across many species and the avian gene, FoxP2 shares remarkable similarities with the human version, with a 100% sequence identity in the DNA-binding domain of the protein (Haesler et al., 2004; Teramitsu et al., 2004). In addition, FoxP2 expression patterns in the songbird brain are similar to that observed in humans and mice with highly enriched expression in the striatum including Area X, the portion of the striatum dedicated to song behavior (Teramitsu et al., 2004). Furthermore, as in the human striatum, FoxP2 expression in Area X is restricted to the medium spiny neurons (MSNs) (Scharff and Haesler, 2005). These findings suggest a conserved function for FoxP2 in human and songbirds.

Consistent with this view, lentiviral shRNA-mediated reduction (LV-ShFoxP2) of FoxP2 in Area X of juvenile male zebra finches is sufficient to disrupt song learning and leads to imprecise and inaccurate imitation of a tutor song (Haesler et al., 2007). FoxP2 knockdown birds produced songs that were abnormally variable, in a manner similar impaired speech production observed with humans with *FOXP2* mutations. Furthermore, FoxP2 knockdown in Area X results in decreased spine density in the MSNs, pointing to a synaptic correlate of these behavioral deficits. These studies provide evidence that deficits in striatal function arising from reduced FoxP2 levels alone are sufficient to disrupt song learning. But these studies do not distinguish the role of FoxP2 in the acute control of song variability from its role in developmentally restricted learning

mechanisms. One possibility is that knockdown of FoxP2 in addition to long-term effects on synaptic organization and song learning could have effects on acute song control. In support of this second idea, humans with *FOXP2* mutations have deficits in acute oral praxis (Watkins et al., 2002a). I hypothesize that FoxP2 knockdown in Area X has acute effects on ongoing song. I used the ability of adult birds to modulate song variability as a function of social context as an assay to distinguish the role of FoxP2 in acute control of song variability from developmentally restricted learning mechanisms (Chapter 2).

On hint for an ongoing role for FoxP2 in adults is the observation that FoxP2 mRNA and protein levels are regulated by the amount of singing in both adult and juvenile birds (Teramitsu and White 2006; Miller et al., 2008; Teramistu et al., 2010). However, while expression of FoxP2 mRNA is lower following undirected singing compared to directed singing in adult birds (Teramitsu and White, 2006), context-dependent differences are not evident in FoxP2 protein levels (Miller et al., 2008). Furthermore, whether these changes are causally linked to song control in adult birds remains unknown. Here I explicitly addressed the issue of causality by reducing FoxP2 protein levels in Area X of adult birds and testing whether this reduction affected their ability to modulate song variability as a function of social context (Chapter 2).

1.7 Signals propagating through the AFP drive song variability

Another distinct advantage of using songbirds to study the role of FoxP2 in learned vocal communication is the potential to link synaptic and circuit properties to behavior. In the singing bird, the song premotor nucleus HVC generates a precise timing signal that is relayed to both RA and Area X (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007; Prather et al., 2008; Long and Fee, 2008; Fujimoto et al., 2011). This precise timing signal then undergoes context-dependent modulation in the AFP, with the consequence that LMAN neurons display increased trial-by-trial variability, higher firing rates and augmented bursting activity during undirected singing relative to directed singing (Kao et al., Brainard, 2005; Kao and Brainard, 2006; Stepanek and Doupe, 2010). These changes in LMAN activity are translated into more variable action potential activity in RA that is correlated with, and thought to drive, greater spectral variability in the bird's song (Sober et al., 2008). Similarly, LMAN neurons exhibit highly variable action potential activity ('spike') patterns and elevated bursting activity in juvenile birds that sing highly variable songs (Olveczky et al., 2005). Finally, electrical microstimulation of LMAN injects acute variability into RA (Kao et al., 2005; Olveczky et al., 2005). These studies support the idea that augmented burst firing and elevated firing rates of LMAN neurons are neural correlates of increased song variability. Therefore, a distinct possibility is that FoxP2 knockdown in Area X results in persistent elevation in LMAN activity during singing. In Chapter 2 of this thesis, I observe that FoxP2 knockdown in Area X abolished context-

dependent song variability, and this effect was attributable to directed songs becoming more variable. I hypothesize that knockdown of FoxP2 in Area X of adult male finches will result in augmented burst firing and elevated firing rates in LMAN neurons during directed singing that in turn drive the increased song variability observed. I tested this hypothesis by performing chronic extracellular recordings of LMAN neurons in adult male zebra finches that had received prior injections of LV-ShFoxP2 while they switched between social contexts (Chapter 3).

Moreover, several studies have suggested that the input-output activities of the AFP are precisely coordinated (Chi and Margoliash, 2001) and it is reasonable to assume that such coordination is important in mediating temporally precise sensory-motor integration required for vocal learning and production. Here, I seek to test the hypothesis that disrupting FoxP2 expression in the Area X alters signal propagation through the AFP. I used *in vivo* intracellular recordings from LMAN neurons while electrically stimulating HVC of anesthetized male zebra finches to test if FoxP2 knockdown in Area X altered signal propagation from HVC to LMAN (Chapter 4).

1.8 Could deficits in dopaminergic modulation underlie FoxP2 mutations?

While HVC sends a precise copy of the timing signal to Area X, how the AFP modulates this precise timing signal it receives from HVC remains unclear. Evidence from recent studies point towards dopaminergic modulation to play an important role in this process. There is an increase in dopamine levels in Area X during directed singing (Sasaki et al., 2006) that correlates with decreased burst firing and firing rates of LMAN neurons. Furthermore, infusion of dopamine receptor 1 (D1R) antagonist into Area X blocks context dependent changes in song variability and infusion of dopamine or a D1R agonist alters signal propagation through the AFP (Leblois et al., 2010). Intriguingly, in embryonic mice several genes implicated in dopamine signaling, including dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), a critical component in D1R-mediated signal transduction machinery are potential targets of Foxp2 regulation (Vernes et al., 2011). In humans, several genes implicated in neuromodulatory signaling pathways, including a gene that encodes an enzyme that metabolizes dopamine (monoamine oxidase B, MAOB), are differentially regulated by FOXP2 in humans versus chimps (i.e., FOXP2chimp) (Konopka et al., 2009; Spiteri et al., 2007; Vernes et al., 2007). Moreover, the knockdown of FoxP2 leads to a decrease in MSN spine density in songbirds (Schulz et al. 2010) and while D1Rs are not thought to be direct targets of FoxP2 regulation, D1Rs have been shown to be concentrated in dendritic spines of medium spiny neurons (MSNs; Levey et al., 1993) and FoxP2 knockdown could lead to an indirect decrease in D1R

concentration in MSNs. These various observations raise the possibility that FoxP2 could regulate dopaminergic modulation of signal propagation through the AFP. To test if impaired dopaminergic modulation underlies striatal deficits observed with FoxP2 mutations I used a combination of *in vivo* intracellular sharp recordings from LMAN neurons and pharmacological manipulations (application of D1R agonists and antagonists) in Area X of anesthetized male zebra finches (Chapter 4). Furthemore, in collaboration with Stephen Haward, we used western blot experiments in to determine if FoxP2 knockdown affected levels of key dopaminergic signaling molecules such as D1Rs and DARPP-32.

1.8.1 Role of dopamine in vocal learning and control

Depleting dopamine levels with drugs, lesions or dopamine receptor antagonists leads to severe deficits in initiation and execution of movements, and is accompanied by changes in basal ganglia activity (Carlsson et al. 1958, Burns et al. 1983, Filion et al. 1988, Schultz et al. 1989, Doudet et al. 1990). Furthermore, deficiencies in dopaminergic signaling in the striatum have been linked to speech deficits associated with several movement disorders such as Parkinson's, Huntington's and Tourette's (Critchley, 1981; Felling and Singer, 2011). Intriguingly, instances of drug-induced acute vocal deficits have been documented in human subjects treated with dopamine antagonists, patients in these studies exhibited supraglottic dystonia, laryngeal spasms, an inability to control tongue movements, and had trouble articulating (Newton-John, 1988; Warren and

Thompson; Incecik et al., 2008). Moreover, dopamine antagonists have been shown to be effective in the treatment of stuttering (Maguire et al., 2001). While these results point to an important role for dopamine in the acute control of speech, the exact neural mechanisms by which dopamine exerts an effect on the acute control of vocal variability in human speech remains to be understood. Studies in songbirds have implicated an important role for dopamine in the acute control song variability. An influx of higher amounts of dopamine into Area X during singing correlates with less song variability (Sasaki et al., 2006). Conversely, infusion of D1R antagonist into Area X blocks the ability of adult male zebra finches to lower their song variability in the presence of a female bird (Leblois et al., 2010). Taken together, these findings demonstrate an important role for dopamine in acute control of variability.

In addition to controlling acute vocal variability, reinforcement models of vocal learning posit an important role for dopamine in encoding reward signals (Doya and Sejnowski, 1995; Fee and Goldberg, 2011). Furthermore, dopamine-mediated synaptic plasticity is believed to play an important role in motor skill learning (Kreitzer and Malenka, 2008), including speech in humans and song learning in birds. In mammalian studies that involve external rewards (e.g. juice rewards in monkeys, food rewards in mice), dopaminergic neurons fire when there is a mismatch between actual and anticipated outcomes (Schultz, 1997, Matsumoto et al., 1999, Tsai et al., 2009). While the evidence for the role of dopamine in tasks that involve external rewards in undisputable,

it still remains unclear if dopaminergic neurons also encode reinforcement signals in the case of intrinsic rewards, e.g. speech learning. One possibility is that in songbirds, VTA neurons play a role in evaluating the bird's own song to the memory of his tutor's song and generating an error signal in the event of a mismatch. In support of this idea, dopaminergic neurons in VTA of songbirds have demonstrated singing related modulation of their activity (Yanagihara and Hessler, 2006; Hara et al., 2007). Furthermore, there is evidence that VTA neurons receive auditory information (and potentially auditory feedback information) from the arcopallium (Gale et al., 2008) and VTA neurons could in turn convey this information to Area X. Furthermore, dopaminergic signaling modulates synaptic plasticity of MSNs (Ding and Perkel, 2004) and regulates signal propagation through the AFP (Leblois and Perkel, 2010).

In summary, there are several lines of evidence pointing to key role for dopamine in the acute control of vocal variability, critical for both speech and song learning. Furthermore, in reinforcement models of song and speech learning, dopamine is believed to play an important role in generating reward signals and modulating striatal plasticity, a key ingredient for motor skill learning. Therefore, deficits in dopaminergic signaling could have acute effects on speech, while also affecting developmentally restricted speech learning mechanisms. This raises the possibility that speech deficits observed in human subjects with *FOXP2* mutations could result from impaired dopaminergic signaling.

1.9 Summary

In summary, while we know reductions in levels of functional FoxP2 protein results in both speech and song deficits, how diminished FoxP2 levels affect vocal control and alter the function of neural circuits important to learned vocalizations remain unclear. I addressed these questions using a combination of behavioral analysis, *in vivo* intracellular recordings in anaesthetized birds, pharmacological manipulations and extracellular recordings in singing birds.

In Chapter 2, I tested the hypothesis that reduction in functional levels of FOXP2, affects the acute control of vocal variability important to vocal learning. I used the ability of adult male zebra finches to modulate their song variability as a function of social context as an assay to understand the role of FoxP2 in the acute control of motor variability. I observed that knockdown of FoxP2 in Area X increased the variability of directed songs.

In Chapter 3 of this thesis, I tested the hypothesis that FoxP2 knockdown abolishes context dependent differences in LMAN activity. More specifically, to identify the neural correlates of increased song variability I used extracellular recordings to monitor how singing related activity of LMAN neurons were altered by FoxP2 knockdown in Area X while the birds switched between social contexts. Recordings in singing birds reveal that FoxP2 knockdown prevents social modulation of singing-related activity of LMAN neurons.

I hypothesize that disrupting FoxP2 expression in the striatum impairs signals propagation times through the AFP and that this impaired signal propagation results from deficits in dopaminergic modulation. In Chapter 4, I used intracellular sharp recordings and pharmacological manipulations to test this hypothesis. Recordings in anaesthetized birds show that FoxP2 knockdown accelerated signal propagation times [2] through the AFP and interferes with D1R-dependent modulation of activity propagation in a corticostriatal pathway important to song variability, an effect that may be partly attributable to reduced D1R and DARPP-32 protein levels (Appendix A). Finally, using a novel electrophysiological slice assay I demonstrated that small differences in signal propagation times through the AFP (~ 3 ms) could alter RA spike timing variability.

[2] Signal propagation times in this study refers to the time taken for a LMAN neuron to respond to HVC stimulation.

2. FoxP2 knockdown in Area X affects acute song variability.

2.1 Introduction

Mutations in the *FOXP2* gene in humans result in severe speech and language deficits. However, because *FOXP2* is highly expressed in the human striatum from embryonic development onwards (Teramitsu et al., 2004), the role of FOXP2 in ongoing vocal motor control cannot be dissociated from a developmentally restricted role in speech learning. The first objective of my thesis was to investigate whether the role of the FoxP2 gene extended beyond developmentally restricted learning mechanisms and to test if reducing levels of FoxP2 in the striatum of songbirds are causally linked to acute song control. I addressed this issue by reducing FoxP2 protein levels in Area X of adult birds that have completed learning their tutor's song and testing whether this reduction affected their ability to modulate song variability as a function of social context. In addition, I also tested whether reducing FoxP2 expression in Area X of the juvenile alters acute levels of song variability during song learning, which in turn could potentially interfere with reinforcement learning mechanisms important to accurate copying of the tutor song.

2.2 Results

2.2.1 Lentivirus shRNA-mediated knockdown is effective in reducing the levels of FoxP2 in Area X of adult male zebra finches

As a first step towards understanding the role of FoxP2 in the control of song variability in adult birds, I used a lentiviral-mediated expression of shRNAs to knockdown the levels of FoxP2 in the Area X of adult male zebra finches. I used two different LV-shRNA constructs (shFoxP2-h-gfp or the shFoxP2-f-gfp; previously used and tested extensively in Haesler et al., 2007) to explore whether song performance is affected by reducing levels of FoxP2 in Area X in adult male zebra finches (> 120 days post hatching, dph), after the process of song copying is complete in this species (Immelmann, 1969). To determine whether these constructs could infect cells and reduce FoxP2 levels in Area X of adult finches, multiple injections of LV-ShFoxP2 or LV-ShControl (LV-ShC) were made bilaterally into Area X using stereotaxic coordinates. Two weeks to four months after the injection, visualization of the GFP marker confirmed extensive expression of the LV-ShFoxP2 constructs (Figure 3).

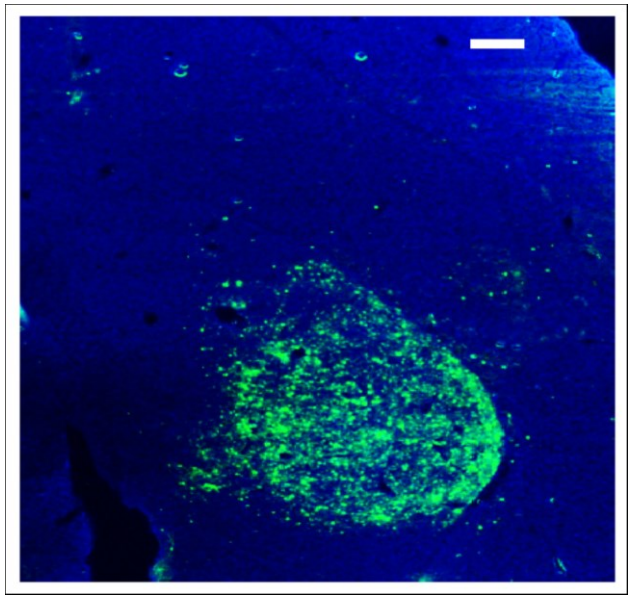

Figure 3: Expression of ShFoxP2-GFP in Area X of an adult male zebra finch.

A parasagittal section through Area X, showing expression of ShFoxP2-GFP (immunoreacted with antibodies to GFP, green fluorescence; DAPI staining in blue) 42 days after the injection of lentivirus-ShFoxP2-GFP into the same region. Scale bar, 100 μm.

To quantify the degree to which these constructs reduced FoxP2 levels, we performed western blots of tissue homogenates obtained from Area X of adult birds previously injected with either LV-ShFoxP2 or LV-ShC. All western blot experiments reported in this thesis were done in collaboration with Stephen Harward.

Optical density measurements indicated that FoxP2 protein levels were reduced by more than two-thirds (~70%) in the LV-ShFoxP2 animals compared to the LV-ShC animals (Figure 4; $n = 12$ birds for each condition, >90 days post injection; $p < 0.05$; significance was assessed using an ANOVA followed by a Student's t-test, unless otherwise noted). This reduction in FoxP2 levels was comparable to that achieved in juvenile birds (Haesler et al., 2007), indicating that the shRNA method is effective at decreasing FoxP2 levels in Area X of adult male zebra finches.

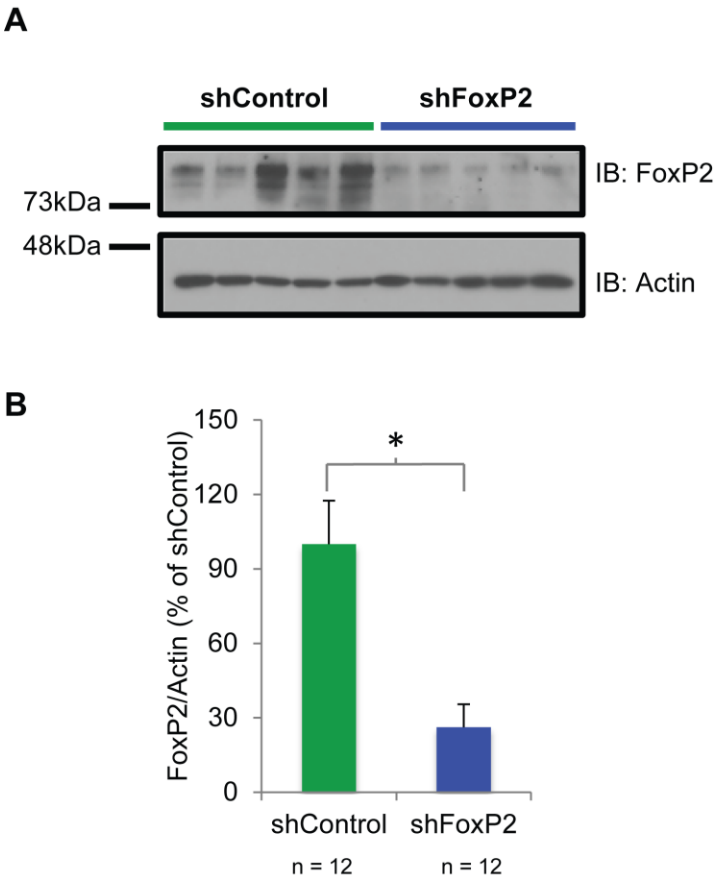

Figure 4: Lentiviral-shRNA mediated knockdown of FoxP2 in Area X of adult male zebra finches

A) A representative immunoblot (IB) showing greater amounts of FoxP2 protein extracted from Area X tissue punches >90 days after the injection of LV-ShC injected birds (left, 5 animals) than after LV-ShFoxp2 injected birds (right, 5 animals). B) Compared to the group of LV-ShC animals, the FoxP2 protein levels in Area X, normalized to actin, were significantly lower in the group injected with LV-ShFoxP2-GFP (mean 73% reduction; $n = 12$ birds in each group, $p < 0.05$).

2.2.2 FoxP2 knockdown in Area X of adults abolishes social context-dependent changes in song variability

I used social context-dependent modulation of song variability in adult birds as an assay to probe whether reducing FoxP2 levels in Area X of adult finches affected song performance independent of song learning. Prior to injecting LV-shRNA into Area X of adult male zebra finches, I confirmed that the pitch of syllables in undirected songs varied more from one trial to the next than the pitch of the corresponding syllables in directed songs (Figures 5A-C; green; Pre-ShFoxP2-undirected versus Pre-ShFoxP2-directed: paired t-test, $p = 0.03$). I then made bilateral injections of either LV-ShFoxP2 or LV-ShC into Area X of these birds and re-evaluated their song performance in different social contexts twenty to thirty days later (ShFoxP2: n = 7 birds, mean age at injection, 127 ± 2 (S.E.M) dph; 26 ± 2 d post injection; ShC: n = 4 birds; mean age at injection, 126 ± 4 dph, 27 ± 2 d post injection). Notably, LV-ShFoxP2 injections in Area X abolished social context-dependent changes in pitch variability (Figure 5B, C; blue; Post-ShFoxP2-undirected versus Post-ShFoxP2-directed: paired t-test, $p = 0.5$), an effect that was specifically attributable to elevated levels of variability in the directed context (Table 1). The mean pitch does not change following the knockdown of FoxP2 (Table 2, $p > 0.05$). In contrast to the knockdown birds, birds that received LV-ShC injections continued to modulate song variability as a function of social context (Figure 5C; red; Post-ShC-undirected vs. Post-ShC-directed: paired t-test, $p = 0.03$).

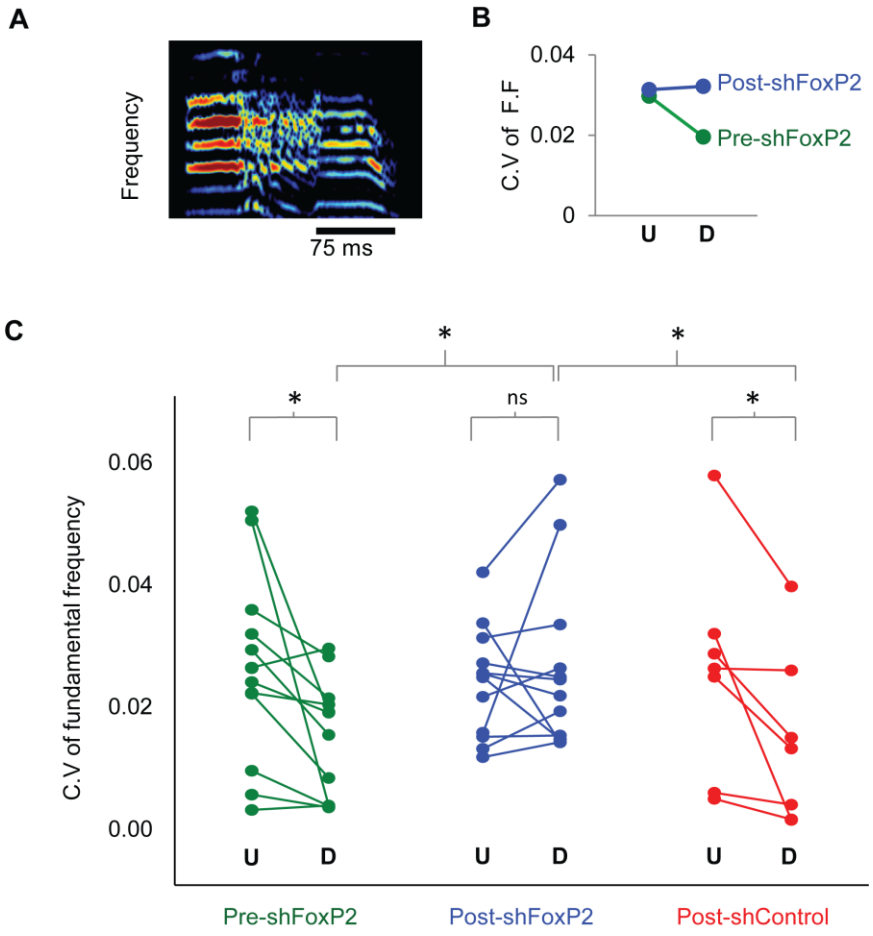

Figure 5: FoxP2 knockdown in Area X of adult zebra finches abolishes context dependent changes in song variability.

A) A sonogram of the type of syllable chosen for analysis of coefficient of variation (C.V) of fundamental frequency (F.F); only the constant frequency portions (i.e., harmonic stacks) were analyzed. Scale bar (black) indicates 75ms, ordinate: 0-9kHz. B) A representative plot of C.V of fundamental frequencies for the syllable shown in A produced during undirected (U) or directed (D) singing pre- (green) and post-injection (blue) of LV-ShFoxP2 into Area X. C) Reducing FoxP2 levels in Area X of the adult abolishes context-dependent differences in syllable variability. In the control conditions (green), the fundamental frequencies of syllables are more variable in the undirected state and become more stereotyped in the directed state ($p = 0.03$). Following knockdown of FoxP2 in Area X (blue, ShFoxP2 n = 7 birds), syllables from the directed songs are more variable, leading to a loss in context-dependent changes in song variability ($p = 0.5$). In contras t, the injection of LV-ShC in Area X (red, ShC n = 4 birds) has no effect on context-dependent changes in song variability ($p = 0.03$).

Table 1: Comparing fundamental frequency (pitch) of syllables in different social contexts and injection conditions.

Comparison	p-value
Pre ShFoxP2 directed versus Post ShFoxP2 directed	p = 0.048
Post ShFoxP2 directed versus Post ShControl directed	p = 0.023
Pre ShFoxP2 undirected versus Pre ShFoxP2 directed	p = 0.033
Post ShFoxP2 undirected versus Post ShFoxP2 directed	p = 0.548
Pre ShFoxP2 undirected versus Post ShFoxP2 undirected	p = 0.874
Post ShControl undirected versus Post ShControl directed	p = 0.031
Pre ShFoxP2 undirected versus Post ShControl undirected	p = 0.890
Pre ShFoxP2 directed versus Post ShControl directed	p = 0.798

Shaded boxes represent $p < 0.05$

Table 2: FoxP2 knockdown does not affect the mean pitch of syllables

Mean Pitch ± s.e.m

Pre ShFoxP2 directed	Post ShFoxP2 directed	Pre ShFoxP2 undirected	Post ShFoxP2 undirected
770 ± 77	771 ± 75	764 ± 71	764 ± 73

All the following comparisons were made in LV-ShFoxP2 animals.

Comparison	p-value
Pre undirected vs Post undirected	0.778
Pre directed vs Post directed	0.771
Pre undirected vs Pre directed	0.372
Post undirected vs Post directed	0.173

p - values are results of paired Student *t*-tests.

2.2.2.1 FoxP2 knockdown has no effect on higher order song features.

In addition to singing songs that are less variable, adult male zebra finches sing songs that are faster, contain more introductory elements per motif and sing more motifs per bout in the presence of a female. However, it appears that these higher order song features are unaffected by LMAN lesions and not regulated by D1R-mediated signaling in Area X (Kao and Brainard, 2006; Leblois and Perkel, 2012; Aronov and Fee 2012). The question remains if FoxP2 knockdown in Area X alters these higher order song features. By measuring these song features in FoxP2 knockdown animals we were able to show that reducing levels of FoxP2 does not affect the ability of birds to modulate song tempo (Figure 6A), the number of introductory elements (Figure 6B), or the number of motifs per bout (Figure 6C, ShFoxP2: n = 7 birds, ShC: n = 4 birds, p = 0.3, ANOVA), all of which are song features that usually change with social context. The absence of context-dependent changes in the number of introductory elements in the control animal and the number of motifs per bout in all groups likely reflect the large variability in these features from bird to bird. Therefore, manipulating FoxP2 levels in Area X of adult birds can increase song variability even after the phase of juvenile song copying is complete while having no effect on higher order song features.

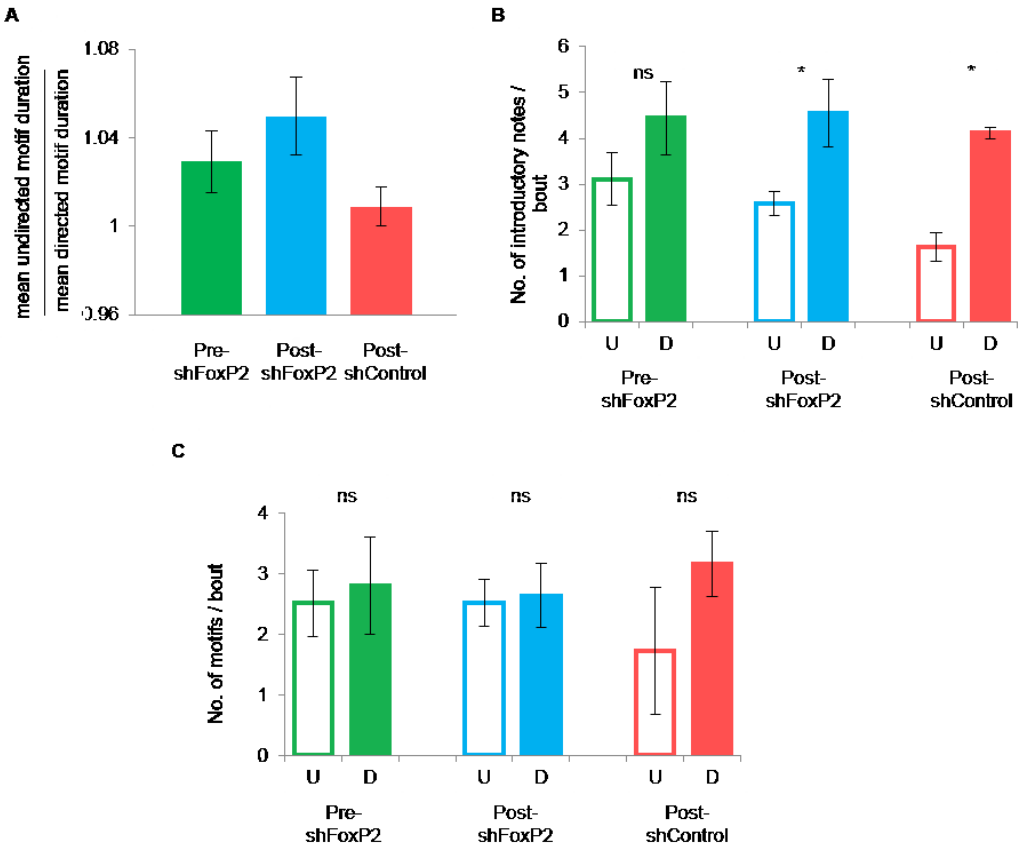

Figure 6: Reducing FoxP2 levels in Area X has no effect on higher order song features that change as a function of social context.

A) Reductions in the level of FoxP2 have no effect on context dependent differences in motif duration ($p = 0.3$, ANOVA, followed by t-test). B) Number of introductory notes in a bout is higher in the directed state (shaded bars) compared to the undirected state (open bars) and reduction in the levels of FoxP2 has no effect on this measure. Bars indicate group means; error bars indicate s.e.m, asterisk denotes $p < 0.05$. C) The number of motifs in a bout is not significantly different in the directed state compared to the undirected state and reduction in the levels of FoxP2 does not alter the number of motifs a bird sings in a bout in either state.

2.2.3 Juveniles with FoxP2 knockdown in Area X display elevated variability throughout learning

Reinforcement models of song learning posit that trial-by-trial variability enables vocal exploration necessary for the accurate copying of a tutor song (Doya and Sejnowski, 1995). A prior study demonstrated that reducing FoxP2 levels in Area X of the juvenile zebra finch impairs its ability to accurately copy a tutor song (Haesler et al., 2007) and we found here that a similar reduction of FoxP2 levels in the adult finch disrupts its ability to modulate song variability in a context-dependent manner (Figure 6, 7). These observations led us to wonder whether reducing FoxP2 expression in Area X of the juvenile alters acute levels of song variability during song learning, which in turn could potentially interfere with reinforcement learning mechanisms important to accurate copying of a tutor song.

To test this idea, I made bilateral injections of either LV-ShFoxP2 or LV-ShC in Area X of juvenile male zebra finches three weeks after hatching (ShFoxP2: n = 6; ShC: n = 4; mean age at the time of injection: 21 ± 1 dph). The juvenile birds were then housed with an adult tutor for a 5-10 day period and their songs were recorded beginning at 45 dph and at regular intervals into adulthood (>90dph). The adult bird's song was used to retrospectively identify the stereotyped syllable sequence, or motif, at various time points in development. As reported previously (Haesler et al., 2007), we observed that birds that received injections of LV-ShFoxP2 in Area X early in juvenile development subsequently sang poorer copies of the tutor song during late juvenile development (65 and 80 dph)

and in adulthood than did birds that had received injections of the control construct (Figure 7). We also noted that the LV-ShFoxP2 birds sang poorer copies of the tutor song at the earliest stage that recognizable song motifs are first produced (i.e., 45-55 dph) (Figure 8). We then measured the variability of the pitch and entropy (i.e., noisiness) of copied syllables at regular intervals throughout development (Figure 9 A, B; ShFoxP2: n = 6 birds, 8 syllables; ShC: n = 4 birds, 6 syllables). Notably, juveniles that had received injections of LV-ShFoxP2 produced syllables that were more variable in their pitch and also noisier compared to those of their LV-ShC injected siblings (Figures 9A-B and 10). Moreover, when we analyzed FoxP2 levels in Area X in a subset of adult birds that had received injections of either LV-ShC or LV-ShFoxP2 early in development (~20dph), we found that FoxP2 levels correlated with tutor song similarity (Figure 11, R = 0.8, n = 5 birds in each group). A strong correlation was not detected in the FoxP2 knockdown animals, likely because 5 birds have comparable levels of knockdown of FoxP2. Whereas, the amount of FoxP2 in Area X of control animals strongly correlated with song learning (R = 0.7). Therefore, reducing FoxP2 levels in Area X of the juvenile affects acute syllable production while also interfering with song learning over a longer time course.

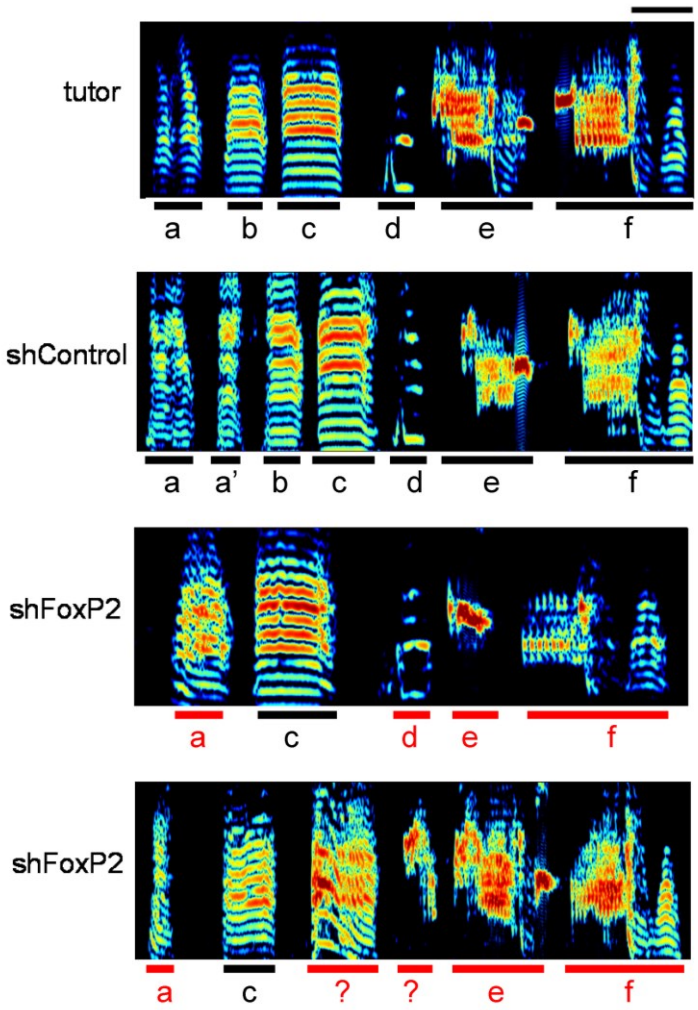

Figure 7: Knockdown of FoxP2 results in imprecise copying of the tutor song

Representative sonograms showing a tutor's song and the adult songs (95 dph) of three of his pupils, including one pupil that received injections of LV-ShC in Area X and two pupils that received injections of LV-ShFoxP2 in Area X early in juvenile life (~20 dph, scale bar indicates 100ms, ordinate: 0-9 kHz). Black bars underline accurately copied individual syllables, red bars underline inaccurately copied syllables.

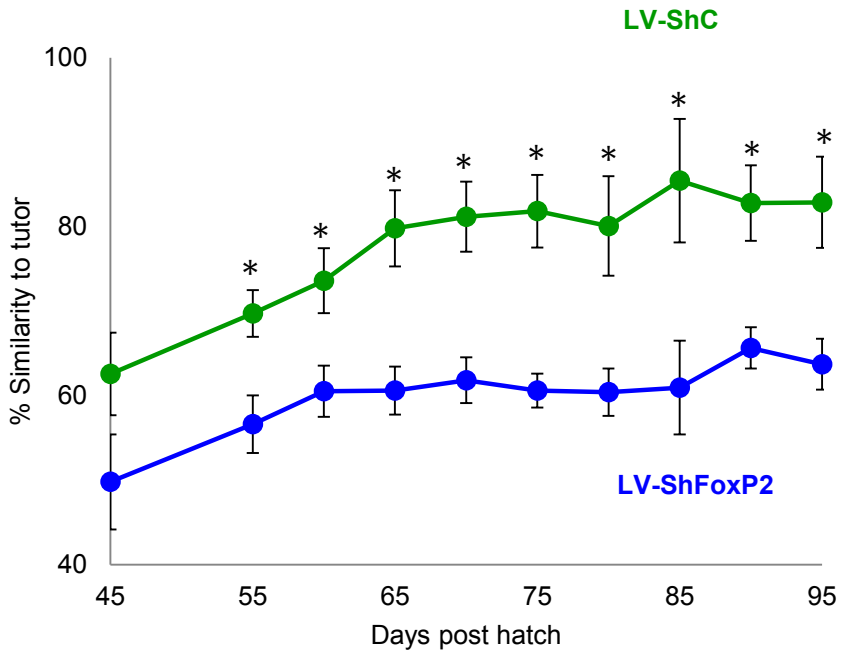

Figure 8: FoxP2 knockdown animals produce poor copies of the tutor song at very early time point in development

Injecting LV-ShFoxP2 (blue, ShFoxP2 n = 6 birds) in Area X at ~20 dph disrupts the ability of juvenile birds to copy a tutor's song, resulting in significantly lower similarity scores to the tutor's song compared to the LV-ShC injected birds (green, ShC n = 4 birds) throughout development (asterisk denotes $p < 0.05$, ANOVA followed by t-test).

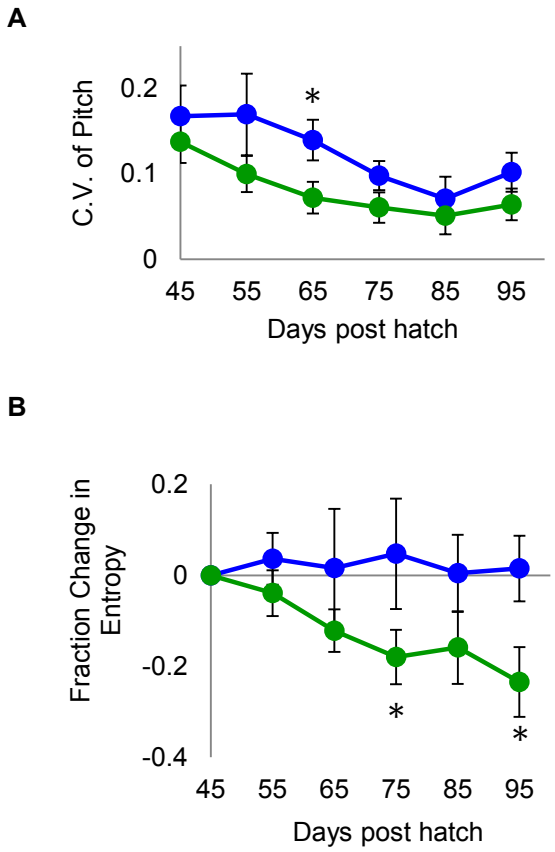

Figure 9: Knockdown of FoxP2 in Area X of juveniles results in elevated song variability throughout song development

A) The coefficient of variation in pitch was higher in the FoxP2 knockdown animals compared to the control animals at 65 dph. B) The entropy of syllables decreased over the course of development in the control animals (green) but not in the knockdown animals (blue). The entropy of each syllable was normalized to its entropy value calculated at 45 dph.

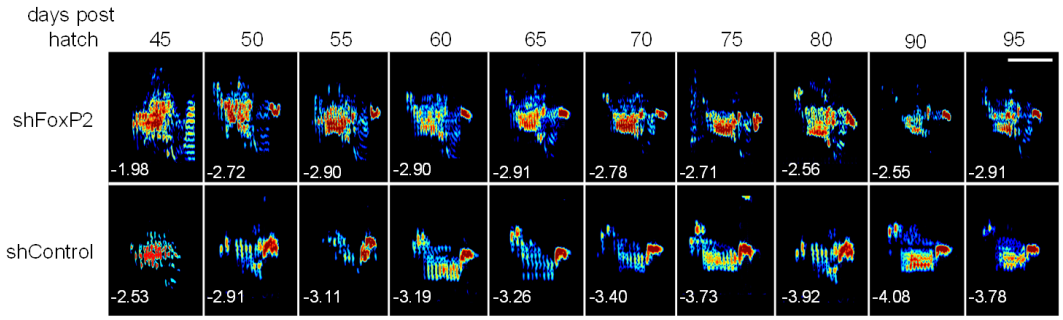

Figure 10: FoxP2 knockdown animals produce noisier syllables through development

Representative spectrograms of a syllable that undergoes a decrease in entropy across development resulting in less noisy syllables following the injection of LV-ShC (bottom panel). In contrast the same syllable produced by a sibling that received injections of LV-ShFoxP2 (top panel) shows significantly higher entropy values over the entire course of development. Values in the panels are mean values for a given day (scale bar indicates 100 ms).

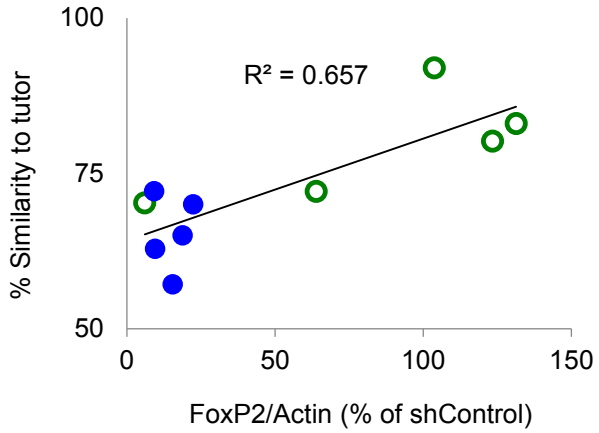

Figure 11: Lower levels of FoxP2 correlate with poorer copies of the tutor song.

Levels of FoxP2 in Area X are correlated with the similarity of the adult bird's song to its tutor's song. (R^2 = 0.65, LV-ShC (green) or LV-ShFoxP2 (blue), n = 5 birds in each group).

To dissociate the effects of LV-ShFoxP2 injections on performance and song development independent of the quality of copying, we compared each bird's song at various stages of development to its adult song. Despite marked differences in their abilities to accurately copy a tutor song, LV-ShFoxP2 and LV-ShC injected animals displayed comparable developmental trajectories to their final adult songs (Figure 12; ShFoxP2: n = 6 birds, ShC: n = 4 birds, $p > 0.05$). These findings are consistent with the idea that reducing FoxP2 levels in Area X of the juvenile causes it to produce syllables that are more variable in pitch and also noisier, raising the possibility that these acute performance deficits could in turn impair the pupil's ability to accurately and precisely copy a tutor song.

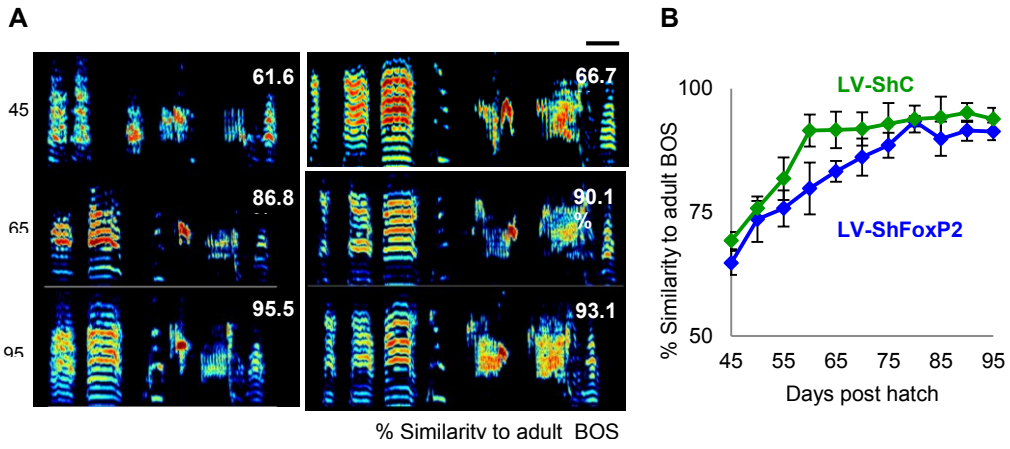

Figure 12: FoxP2 knockdown and control animals have comparable learning trajectories

A) Representative sonograms of songs produced by an LV-ShFoxP2-injected bird (left) and an LV-ShC injected (right) bird recorded at 45, 65, and 95 dph (numbers in white represent average % similarity to the adult BOS for the given day, scale bar indicates 100 ms). B) Comparing each bird's song recorded at various time points across development to its adult song reveals that both control (green) and FoxP2 knockdown birds (blue) display comparable developmental trajectories.

2.3 Discussion

FOXP2 mutations in humans result in severe speech and language deficits. However, understanding the nature of these behavioral deficits in humans is challenging for multiple reasons. First, since the *FOXP2* gene is expressed in humans from embryonic development onwards, it is impossible to dissociate the role of gene in acute vocal control from developmentally restricted learning mechanisms. Second, since the FOXP2 gene in humans in expressed in multiple brain regions including the striatum, cerebellum, thalamus and the deep layers of the cortex, attributing the speech and language deficits to dysfunction in specific brain areas and circuits has been particularly challenging.

In this chapter, I used a lentiviral shRNA-mediated approach to reduce FoxP2 protein levels in Area X of adult male zebra finches. Using social context-dependent modulation of song variability as an assay, I was able to demonstrate that reducing FoxP2 levels in Area X of adult birds abolishes their ability to modulate song variability as a function of social context. This finding dissociates the effects of FoxP2 knockdown on ongoing adult motor (vocal) control from effects during juvenile learning and rules out a model where this gene only contributes to juvenile song learning and extends its role to encompass the adult's ability to generate appropriate behavioral responses to salient social cues.

Prior studies have shown that social context-dependent changes in song variability are driven by changes in LMAN activity (Kao et al., 2008) in a dopamine dependent

process (Sasaki et al., 2006; Leblois et al., 2010). Furthermore, several molecules implicated in D1R-mediated signaling, including DARPP-32, have been implicated to be a downstream target of Foxp2 (Vernes et al., 2011). Our findings combined with these prior studies raise the possibility that loss of social context-dependent changes in song variability in the FoxP2 knockdown animals is due to loss of context-dependent changes in LMAN activity and are resultants of impaired dopaminergic signaling. I tested these ideas in Chapter 3 and Chapter 4 of my thesis respectively.

Furthermore, in this chapter I was able to demonstrate that juvenile birds that received LV-ShFoxP2 injections in Area X produced syllables that were more variable in pitch and noisier in comparison to those produced by their control-injected counterparts. This raises the possibility that acute performance deficits could in turn impair the pupil's ability to harness performance variability that is theorized to play an important role in reinforcement models of song learning.

2.4 Methods

All experimental procedures were conducted in accordance with the National Institutes of Health guidelines and were reviewed by the Duke University Institutional Animal Care and Use Committee (IACUC). Songs were recorded in sound attenuation chambers with microphones (Shure SM 93), pre-amplified and saved to a computer using Sound Analysis Pro (Tchernichovski et al., 2000).

2.4.1 Subjects

2.4.1.1 Adult behavioral experiments

For experiments examining the effect of knockdown of FoxP2 on context-dependent singing, adult male zebra finches (> 120 dph; ShFoxP2: n = 7 birds, mean age at injection, 127 ± 2 (S.E.M) dph; ShC: n = 4 birds; mean age at injection, 126 ± 4 dph) were placed in recording chambers.

2.4.1.2 Juvenile behavioral experiments

For experiments examining the effect of knockdown of FoxP2 on juvenile song development, juvenile male zebra finches raised with their nesting group were injected with either LV-ShFoxP2 (n = 4 birds) or LV-ShC (n = 2 birds) at ~20 dph. In a parallel set of experiments, juvenile male zebra finches were isolated from adult male birds between 7-10 dph and injected with either LV-ShFoxP2 (n = 2 birds) or LV-ShC (n = 2 birds) at ~20 dph. Birds raised in their nesting groups and those isolated between 10-40 dph from male birds showed comparable learning in their respective categories (ShFoxP2 groups, $p = 0.4$ and ShC groups, $p = 0.2$, t-test) and were therefore grouped together for data analysis.

2.4.2 Virus Injections

Birds were anaesthetized with 1-2% isoflurane and placed in a stereotaxic setup. Lidocaine (Fougera) was used as a local anesthetic and the scalp was dissected along the midline. The coordinates for Area X (5.3 mm rostral, 1.6 mm lateral and 2.7-3 mm in depth) were measured from the midsagittal sinus bifurcation at a 45° head angle. Multiunit recordings

were performed using Carbostar-1 (Kation Scientific) to map out the extent of Area X. A glass pipette attached to a Nanoject-II (Drummond Scientific) was used to deliver an overall volume of ~1 μL in each hemisphere. An average of 4-6 sites were targeted per hemisphere in order to span the entirety of Area X, with each site receiving no more than 200 nl of the virus at a rate of 32.2 nL every 30 s. The shFoxP2 constructs were packed into lentivirus particles to yield a final titer of $1x10^7$ to 10^9 particles / mL (Marguerita Klein, Duke University; Roberts et al., 2008). The lentivirus particles expressing either U6-shFoxP2-h-ubiquitin-gfp or the U6-shFoxP2-f-ubiquitin-gfp were used interchangeably to knockdown FoxP2 expression in Area X (Haesler et al., 2007). Control lentiviral shRNA (shControl) particles (Santa Cruz Biotechnology, sc-108080) were used for the control experiments. All behavioral and electrophysiological experiments were carried out > 15 days post injection.

2.4.3 Song recording and analysis

Sound Analysis Pro (SAP) was used to analyze all behavioral data. All song files were bandpass filtered between 400 Hz and 10 kHz.

2.4.3.1 Adult behavioral experiments

For the adult experiments, recordings of each bird were obtained both 2 ± 1 days pre and 27 ± 1 day post-injection of either LV-ShFoxP2 or LV-ShC. Undirected songs were classified as those songs recorded when male birds were isolated in the recording chambers. In order to obtain directed songs, one or two female birds were introduced into

the chamber in a separate cage. A webcam was used to monitor the behavior of the male birds. Those songs in which the male bird faced the female bird(s) were classified as directed songs. Directed singing was restricted to a 2 min period after the introduction of the female bird and was terminated by her removal. The process was repeated at 1-hour intervals until a minimum of 20 directed and undirected songs were acquired.

Only syllables with constant frequency components (i.e., harmonic stacks) were included in the analysis (Kao and Brainard, 2006). The syllables were manually selected using SAP. The fundamental frequency (FF) of harmonic stacks was measured for songs rendered in both directed and undirected conditions. To calculate the coefficient of variability, the FF of a minimum of 20 renditions of each syllable was used. For the tempo analysis, the duration of the complete motifs were measured in in both directed and undirected states for each bird and the mean duration calculated (Kao and Brainard, 2006). The number of introductory elements per bout was counted backwards from the first syllable of a motif till the presence of a 500ms silent period or a call (Kao and Brainard, 2006). A bout was defined as motifs separated by silent periods > 500 ms. To count the number of motifs per bout, motifs that contained more than half the number of syllables of a complete motif were included in the analysis. For all the above analysis, a minimum of 20 bouts per condition (directed and undirected) per bird was used for analyses.

2.4.3.2 Juvenile behavioral experiments

Following the injection, juvenile birds raised in their nesting groups were returned to a sound-attenuating chamber with an adult male tutor (their father) until 45 dph. In the case of the isolated juvenile birds, they were introduced to an adult male zebra finch for five days of tutoring between 45-50dph. Following the tutor experience all juvenile birds were moved into isolated sound-attenuating chambers and their songs were recorded between 45-95dph (all songs were recorded in the undirected state). For the juvenile experiments, 50 renditions of the motifs and syllables were chosen for any given day of analysis. To measure the amount of song learning at any given time point, 50 renditions of the pupil's motif from that day were compared to a single representative motif from his tutor. The percentage similarity score (asymmetric comparisons) was used to quantify the amount of song learning. The percentage similarity score (% similarity) takes into consideration multiple features of song such as pitch, amplitude modulation, frequency modulation, Wiener entropy and goodness of pitch over the course of a motif and determines how these features of the pupil's song compare to the song of the tutor. Multifactor ANOVA followed by post-hoc t-tests were used to determine if the amount of learning differed between the knockdown birds (n = 6) and control birds (n = 4) throughout development. For the syllable level analysis, the syllables were restricted to those that were copied by both the FoxP2 knockdown juveniles and their control siblings. The FF of harmonic stacks and Wiener entropy of syllables were calculated at multiple time points through

development (Tchernichovski et al., 2001). For the entropy analysis, the entropy at all the different time points was normalized to the entropy of the same syllable at 45 dph. Therefore, a more positive value for fraction change in entropy indicates a noisier syllable.

For comparison to the bird's own song (BOS), the songs of juvenile male zebra finches at multiple points during development were compared to the BOS at 95 dph. The analysis is identical to the similarity comparisons to the tutor's song.

2.4.4 Western Blots

All animals were placed in a dark chamber without light for 2 hours starting around noon. The birds had received injections of either LV-ShFoxP2 or LV-ShControl ~ 90 days prior to retrieval of brain samples. The birds were anesthetized with 5% isoflurane and decapitated. Their brains were quickly removed from the skull and were mounted on a vibratome (Leica, VT 1000s) stage. Area X was previously determined to be ~ 1200 μm from the rostral tip of the brain, and 100 μm sections were cut until Area X was visible (Miller et al., 2008). A 0.8mm tissue punch was used to remove much of the tissue encompassing Area X. GFP fluorescence from the virus injection was used to confirm the location of Area X. Only samples from the left hemisphere were used for the western blot experiments. The samples were quickly frozen in liquid N2 and stored at -80° C until later use. Frozen tissue was homogenized in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate), centrifuged at 16000g for 10 min, and supernatant collected. This supernatant was defined as tissue homogenate and

was resolved with SDS-Page. The blots were incubated with FoxP2 (1:500, Sigma-Aldrich) and actin (1:10000, Sigma-Aldrich) primary antibodies for at least 12 hours at 4°C followed by incubation with secondary antibodies (1:5000, Jackson Labs) for at least 1 hour at room temperature. For quantitative analysis, immunoblots were scanned with a digital scanner and the optical band density was quantified using ImageJ analysis software. Optical densities for FoxP2, D1R, and DARPP-32 were normalized to corresponding actin levels. Data are presented as mean ± SEM. Statistical significance was assessed with the Student's t-test.

2.4.5 Immunohistochemistry

Birds were anesthetized with 0.08 ml equithesin (i.m. injection) and transcardially perfused with 0.025 M phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde in PBS (PFA). Brains were dissected out and post-fixed in 4% PFA with 30% sucrose overnight at 4° C. Parasagittal sections were cut on a freezing microtome (Reichert) at 50-75 μm. A GFP antibody staining was run to enhance visualization of the knockdown. The primary antibody used was a mouse monoclonal anti-GFP (Invitrogen, 1:1000 dilution) followed by a goat anti-mouse secondary antibody coupled to Alexa 488 (Invitrogen, 1:500 dilution).

2.4.6 Statistics

Single or two-factor ANOVA was used to test for statistical significance followed by post-hoc Student t-tests. Reported errors are standard errors of mean (s.e.m).

3. FoxP2 knockdown in Area X of adults abolishes context-dependent changes in LMAN activity

3.1 Introduction

FoxP2 knockdown impairs the ability of adult male zebra finches to down regulate song variability in the presence of female birds. However the neural correlate of this increased variability following FoxP2 knockdown remains unknown. Prior studies have implicated LMAN to play an important role in generating acute song variability (Kao et al., 2005; Kao and Brainard, 2006). Furthermore, LMAN neurons exhibit social context-dependent changes in neural activity, they switch from higher trial-by-trial variability, increased firing rates and augmented bursting activity during undirected singing to lower trial-by variability, lower firing rates and diminished bursting during directed singing (Kao et al., 2008).

I hypothesized that knockdown of FoxP2 in Area X of adult zebra finches abolishes context-dependent changes in LMAN activity and that in turn abolishes context dependent differences in song variability. In Chapter 3, I test this using chronic extracellular methods to record the singing-related activity of LMAN neurons in knockdown and control birds while they switched between social contexts. In addition, I was also able to simultaneously monitor changes in song behavior in these birds.

3.2 Results

3.2.1 FoxP2 knockdown abolishes context dependent changes in LMAN activity

To test whether FoxP2 knockdown in Area X abolishes context-dependent changes in the singing-related activity of LMAN neurons, we recorded the singing-related activity of LMAN neurons in adult male zebra finches that had previously received injections of LV-ShFoxP2 in Area X (n = 3 birds; 110 ± 8 dph at implantation; injections were made 32 ± 8 days prior to electrode implantation; see Methods) and control animals. In contrast to LMAN neurons in the control animals, the mean firing rate, trial-by-trial variability and levels of bursting activity in LMAN neurons remained elevated in LV-ShFoxP2 animals as they switched from undirected to directed singing (Figures 13-16, blue; song analysis confirmed that these animals did not exhibit context-dependent changes in song variability, Figure 17). Consequently, the ISI distributions of LMAN neurons recorded in the directed state were left-shifted in FoxP2 knockdown animals relative to controls (Figure 15 K-S test, p = 4.4381e-19). Notably, the ISI distributions from LMAN neurons recorded in the undirected state were also left-shifted in knockdown animals relative to controls (Figure 15, p = 0.003, K-S test). Furthermore, both the mean firing rates and levels of bursting activity of LMAN neurons recorded in the directed state were significantly higher in the LV-ShFoxP2 animals (Figure 14 A and B; p = 0.006 and p = 0.013 respectively). Finally, FoxP2 knockdown abolished during context-dependent changes in trial-by-variability of LMAN activity (p = 0.65), with activity patterns during directed singing

becoming more variable compared to control animals (Figure 16 A and B; $p = 0.01$). Therefore, reducing FoxP2 levels in Area X abolishes context-dependent changes in both LMAN singing-related activity and song variability.

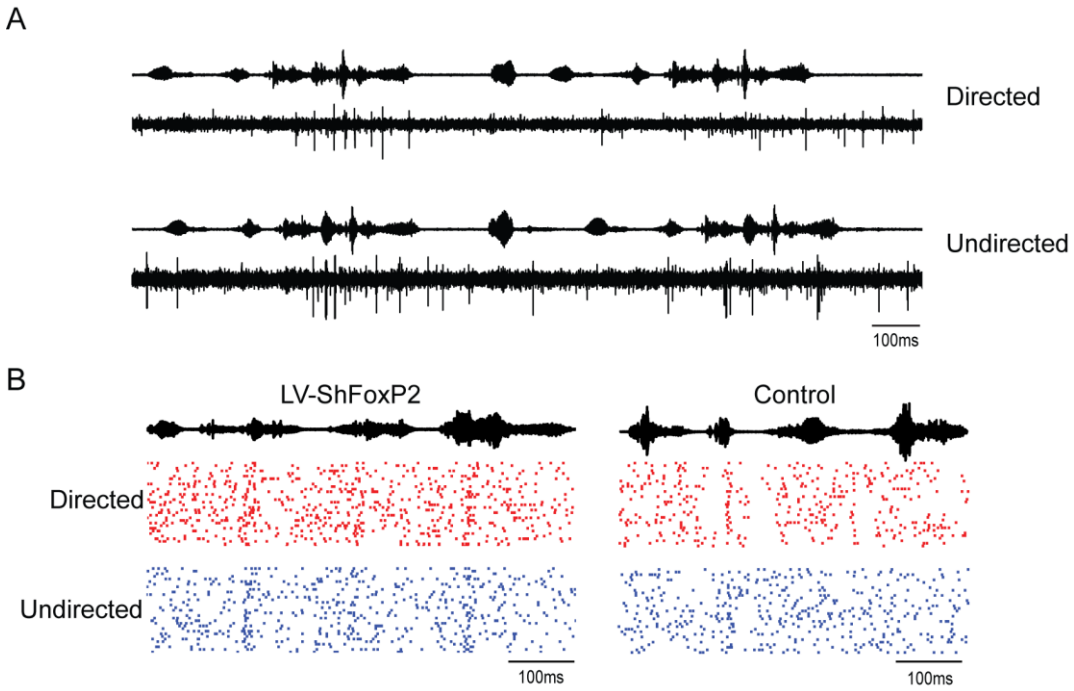

Figure 13: Knockdown of FoxP2 abolishes context dependent differences in LMAN activity

A) An example of singing-related multiunit activity recorded in LMAN of a normal adult male zebra finch when he sings to a female (directed songs; top) and when he sings alone (undirected songs; bottom). Amplitude envelopes of the sound recordings are plotted above the neural traces; the bird sang two consecutive motifs in each context. Spike sorting of the multiunit records was used to isolate single units and measure mean firing rates and inter-spike intervals (see Methods). B) Example raster plots of singing related activity in sorted LMAN units from a LV-ShFoxP2 (left) and control animal (right) as they switched between social contexts (34 trials in each condition). Each dot signifies a spike and each line represents a trial.

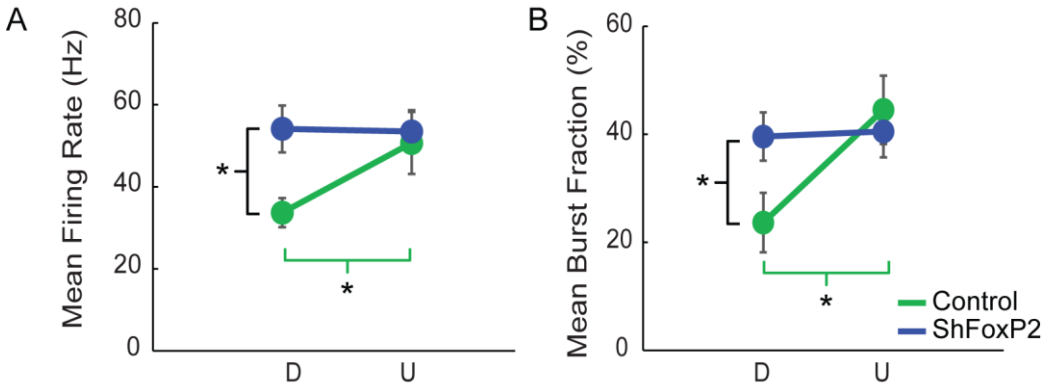

Figure 14: Knockdown of FoxP2 eliminates context-dependent differences in burst firing and firing rates of LMAN neurons

A) In control animals, the mean firing rate of LMAN neurons (n = 9 units, 3 birds) is significantly higher in the undirected state compared to the directed state (green; p = 0.009; paired t-test). In contrast, in the LV-ShFoxP2 animals, LMAN neurons (n = 20 units, 3 birds) display comparable firing rates in the two states (blue; p = 0.859; paired t-test). The mean firing rate of neurons in the LV-ShFoxP2 animals is significantly higher compared to control animals in the directed state (p = 0.006), while the mean firing rates in the undirected state are comparable (p = 0.707). B) In control animals, the mean burst fraction (%) is significantly higher in the undirected state compared to the directed state (green; p = 0.002, paired t-test). This context-dependent difference in mean burst fraction was absent in LV-ShFoxP2 animals (blue; p = 0.575, paired t-test). The mean burst fraction is significantly higher in the LV-ShFoxP2 animals compared to the control animals in the directed state (p = 0.01), but not the undirected state (p = 0.546).

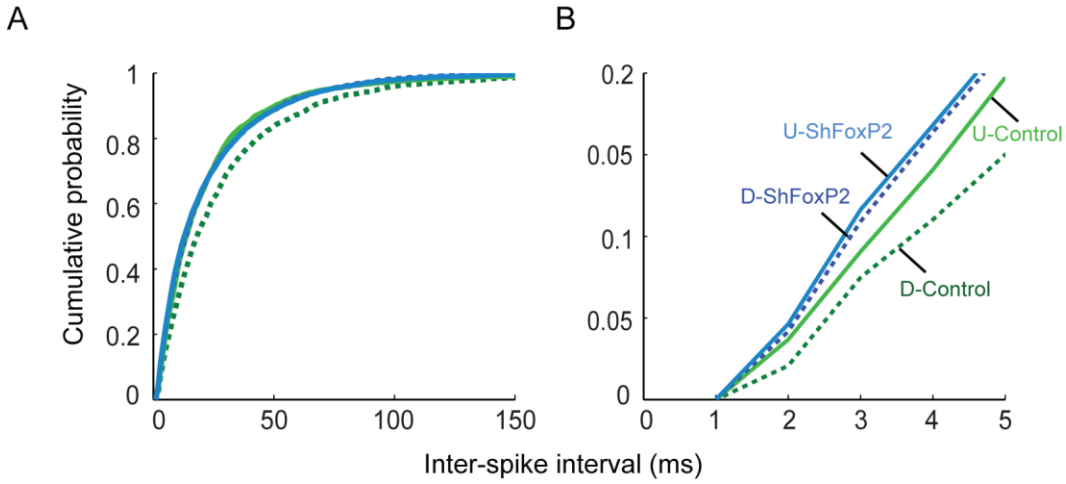

Figure 15: The knockdown of FoxP2 leads to significantly smaller interspike intervals

A) Cumulative probability distribution of inter-spike intervals (ISI) of singing-related activity of LMAN neurons recorded in LV-ShFoxP2 animals are indistinguishable between the directed (D, dotted) and undirected states (U, solid) (blue: $p = 0.127$, K-S test). In contrast, the ISI distribution of LMAN neurons recorded in control animals is significantly left-shifted in the undirected state compared to the directed state (green: $p = 9.413$ e-13, K-S test). The ISI distributions of LMAN neurons recorded from control and LV-ShFoxP2 animals are significantly different in the directed state ($p = 4.4381$ e-19, K-S test) and in the undirected state ($p = .003$, K-S test). B) The panel on the right shows ISI distributions over the interval that corresponds to bursting activity in LMAN neurons (i.e., 1-5 msec, or > 200 Hz).

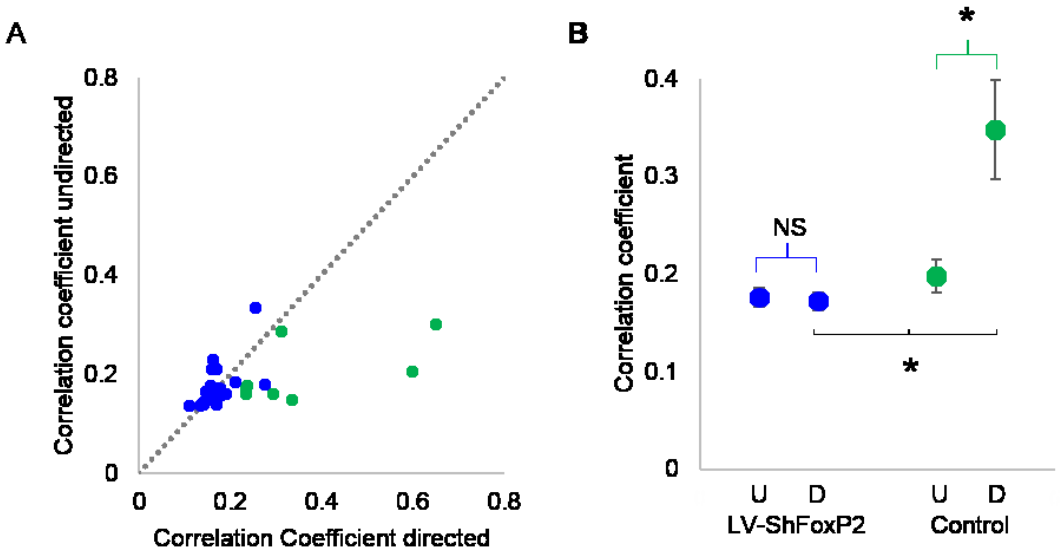

Figure 16: Knockdown of FoxP2 abolishes context dependent changes in trial-by-trial variability of LMAN neurons

A,B) In control animals, LMAN neurons (n = 3 animals, 9 units) exhibit lower trial-by-trial variability (correlation coefficient across trials) in firing patterns during directed singing relative to undirected singing (blue; p = 0.01). In contrast, FoxP2 knockdown abolishes context-dependent difference in trial-by-trial variability (green, p = 0.65). LMAN neurons recorded in the FoxP2 knockdown birds had higher trial-by-trial variability during directed singing compared to the control animals (p = 0.01).

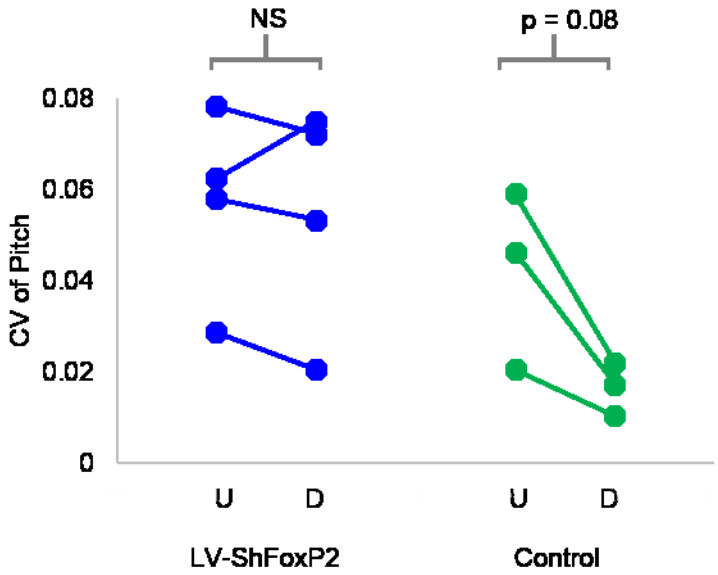

Figure 17: Knockdown of FoxP2 abolishes context- dependent changes in LMAN activity and song variability

FoxP2 knockdown in Area X of the adult abolishes context-dependent differences in syllable variability (blue, n = 4 syllables, 3 birds; p = 0.626) and these changes happen in parallel to abolishing context-dependent changes in LMAN activity. In the control conditions (green), the fundamental frequencies of syllables are more variable in the undirected state and become more stereotyped in the directed state (n = 3 syllables, 3 birds; p = 0.08).

3.3 Discussion

In Chapter 1, I had demonstrated that knockdown of FoxP2 abolishes context-dependent modulation of song variability. FoxP2 knockdown animals sang more variable directed songs compared to the control animals. In order to understand the neural correlates that underlie this increased song variability, I used chronic extracellular methods to record the singing-related activity of LMAN neurons in knockdown and control birds while they switched between social contexts.

A prior study has shown that LMAN neurons in control animals switch from a state of augmented burst firing and higher firing rates while singing undirected songs to a state of reduced burst firing and lower firing rates during directed singing, a finding that I was able to confirm in this Chapter. Importantly, we observed that knockdown of FoxP2 abolished these context dependent differences in LMAN activity. LMAN neurons recorded in the FoxP2 knockdown animals maintain augmented burst firing and elevated firing rates while singing directed songs. Moreover, we were able to show that abolition of context-dependent changes in LMAN activity happened in concurrence with the abolition of context-dependent changes in song variability.

These findings strongly suggest that augmented burst firing and elevated firing rates of LMAN neurons underlie the increased song variability in directed songs observed in the FoxP2 knockdown animals. While, these studies have identified abolition of context-dependent changes in LMAN activity as the neural underpinnings of increased

song variability, the nature of the striatal deficits that result in impaired vocal performance and learning remain unclear.

Prior studies have shown that in zebra finches dopamine levels in Area X change with social context and in embryonic mice several genes implicated in dopamine signaling are potential targets of Foxp2 regulation (Sasaski et al., 2006; Vernes et al., 2011). These various observations raise the possibility that FoxP2 could regulate dopaminergic modulation of signal propagation through the AFP and that impaired dopaminergic signaling could underlie striatal deficits that result from the knockdown of FoxP2. We tested this possibility in Chapter 4 of this thesis.

3.4 Methods

3.4.1 *In vivo* extracellular recordings in singing birds

A custom built manually (threaded rod) operated microdrive carrying an insulated platinum electrode (1-5 MΩ) was stereotaxically implanted in LMAN in an anaesthetized adult male zebra finch. LMAN was located in the anaesthetized animal using stereotaxic coordinates and by its characteristic bursting activity, large units with narrow spike widths (~1ms). After LMAN was located the electrode was withdrawn to a position 200 μm dorsal to LMAN. A reference electrode, consisting of an uninsulated platinum wire, was positioned 1mm posterior to the recording electrode and a ground electrode, made of uninsulated silver wire, was placed over the cerebellum. The microdrive, electrodes and ground wire were secured to the bird's skull using dental cement. All recordings were

limited to the right hemisphere. After the animal was fully recovered (~4 days post implant) the electrode was lowered slowly in ~25 μm steps every 1 hour until LMAN activity was detected. The electrode signal was amplified through a JFET on the cable attached to the bird's head and an op-amp followed by an instrumentation amplifier (Brownlee Precision, Model 440), filtered between 200 Hz and 10 kHz. All electrophysiological and song data were collected inside a sound-attenuating chamber (Industrial Acoustics) using a data acquisition board (National Instruments) controlled by custom MATLAB software (K.Hamaguchi). A video camera was used to observe the bird's behavior. Multinunit data were collected in interleaved trials of directed and undirected singing. The data were visually inspected to exclude trials that had movement artifacts. Spike sorting on the multiunit data was performed offline using Wave clus (Quiroga et al., 2004). The parameters on Wave Clus were set to identify spikes that were a minimum of 3 standard deviations from baseline and the detector dead time was set as 2ms. The waveforms of the sorted clusters were visually inspected and tested for refractory period violations (<1% of interspike intervals ≤ 1 ms; Control: n = 9 units from 3 animals; LV-ShFoxP2: n = 20 units from 3 animals). All sorted single unit data were aligned to the onset of the first syllable of the song motif without time warping. Bursts were defined as spikes separated by less than 5ms. Burst fraction was defined as the fraction of the total spikes that occur as bursts. Mean firing rate and burst fraction were

calculated over the entire duration of the motif. Data were analyzed using custom MATLAB scripts.

For analysis of trial-by-trial variability, first, the onset and offset times for each syllable in a motif were measured. A reference motif was selected for each bird and was kept constant for both conditions (directed and undirected). The data were time warped such that each syllable and the corresponding neural data was stretched or compressed to the reference syllable in the standardized motif (as described in detail by Kao et al., 2008). The instantaneous firing rates were generated for each time-warped unit by smoothing spike trains with a Gaussian filter (10 ms standard deviation). A linear pairwise correlation analysis was run on the instantaneous firing rates comparing all trials a given condition. This values generated was then used to calculate the average correlation coefficient for each unit for a given condition. All data are represented as mean ± ANOVA followed by post t-test were used to test for statistical significance.

3.4.2 Song recordings and analysis

All song data were collected inside a sound-attenuating chamber (Industrial Acoustics) using a data acquisition board (National Instruments) controlled by custom MATLAB software (K.Hamaguchi). A video camera was used to observe the bird's behavior. An average of xx renditions per condition (directed and undirected) per bird was used for analyses. Song data was analyzed using SAP as described in Chapter 2. Student *t*-tests were used to test for significance.

3.4.3 Statistics

Single or two-factor ANOVA was used to test for statistical significance followed by post-hoc Student t-tests for the comparisons of the mean firing rate and percentage mean burst fractions. The non-parametric Kolmogorov–Smirnov test was used to compare if the ISI distributions were different.

4. Reducing FoxP2 expression in Area X alters signal propagation through the AFP

4.1 Introduction

Knockdown of FoxP2 in Area X of songbirds results in the increased song variability resulting from augmented burst firing and elevated firing rates of LMAN neurons during directed singing. How altered FoxP2 expression impacts transmission through corticostriatal circuits ultimately to affect learned vocalizations remains unknown.

HVC generates a precise timing signal that is relayed to both RA and Area X. How the AFP modulates the precise timing signal it receives from HVC is unknown, but in zebra finches dopamine levels in Area X change with social context and in embryonic mice several genes implicated in dopamine signaling are potential targets of Foxp2 regulation (Sasaski et al., 2006; Vernes et al., 2011). These various observations raise the possibility that FoxP2 could regulate dopaminergic modulation of signal propagation through the AFP.

In this chapter, I seek to test the hypothesis that disrupting FoxP2 expression in the striatum impairs signals propagation times through the AFP and that this impaired signal propagation results from deficits in dopaminergic modulation. I used a combination of *in vivo* intracellular physiology from LMAN neurons, pharmacological manipulations in Area X of anesthetized male zebra finches and slice physiology to determine how knockdown of FoxP2 affects signal propagation through the AFP.

4.2 Results

4.2.1 Knockdown of FoxP2 accelerates signal propagation through the AFP

However, the relationship between the modulation of singing-related activity in the AFP and song variability raises the possibility that reduced FoxP2 levels in Area X could affect signal propagation from HVC to LMAN. To test this idea, we made sharp electrode intracellular current clamp recordings from LMAN neurons in diazepam-anesthetized male zebra finches previously injected in Area X with LV-ShFoxP2 or LV-ShC, and applied a brief pulse of electrical current through a bipolar stimulating electrode positioned in the ipsilateral HVC (Figure 18A; 40 μA, 400 μs, see Methods; LV injections were made ~20 dph). Electrical stimulation in HVC elicited excitatory synaptic responses in all (72/72) LMAN neurons, regardless of whether they were recorded from LV-ShFoxP2 or LV-ShC animals, indicating that reduced FoxP2 levels in Area X does not prevent signal propagation through the AFP. However, the latency to the onset of the evoked synaptic response was significantly shorter in the LV-ShFoxP2 animals, indicative of faster conduction times through the AFP (Figures 18B, C; synaptic latency: ShFoxP2 = 12.5 ± 0.3 ms; ShC = 15.5 ± 0.3 ms; ShFoxP2: n = 35 cells, 5 birds; ShC: n = 37 cells, 4 birds; $p < 10E-5$). In contrast, the mean amplitude and coefficient of variation of latency of the evoked synaptic responses did not differ between LMAN neurons from LV-ShFoxP2 and LV-ShC animals (Figures 19A and B). Furthermore, there were no significant differences in the interspike interval (ISI) distributions and spontaneous firing rates of LMAN neurons in

the two groups (Figures 20A and B). Therefore, reducing levels of FoxP2 in Area X specifically affects how rapidly signals propagate from HVC through the AFP.

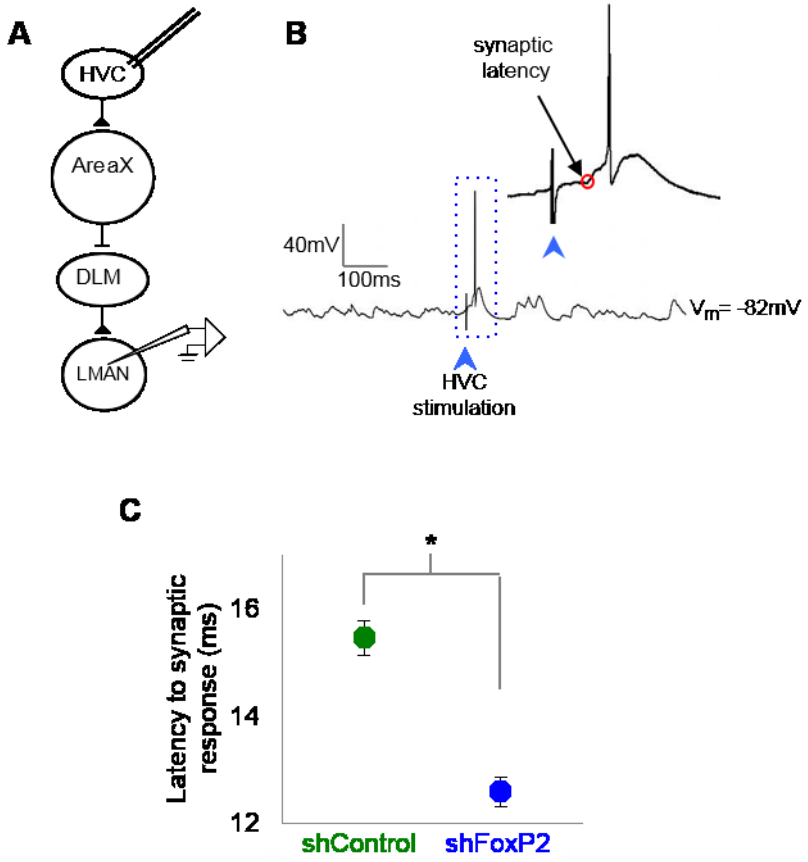

Figure 18: Knockdown of FoxP2 accelerates signal propagation times through the AFP.

A) A schematic showing the placement of the stimulation and recording electrodes. B) *In vivo* intracellular membrane potential recording from an LMAN neuron showing the synaptic response evoked by brief electrical stimulation in HVC (40 µA, 400 µs, bipolar electrodes). C) Signal propagations times (synaptic latency) from HVC to LMAN are shorter in LV-ShFoxP2- than in LV-ShC-injected birds ($p < 0.001$, ShFoxP2 n = 37 cells, ShC n = 35 cells).

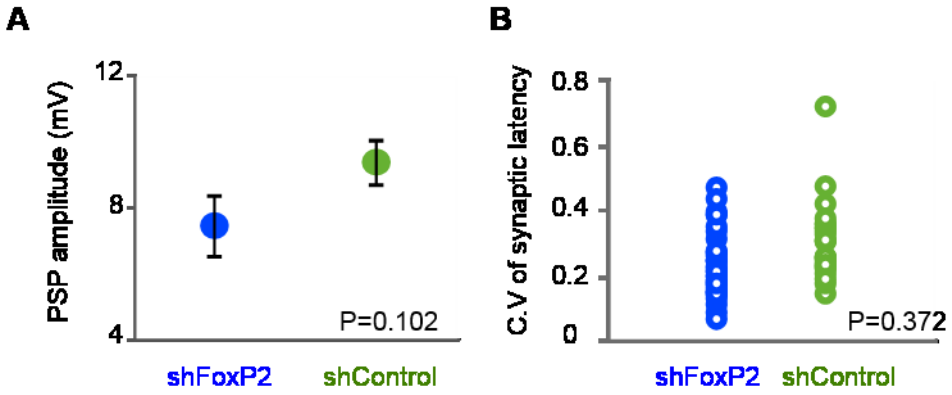

Figure 19: Knockdown of FoxP2 has no effect on other features of signal propagation features

A) Knockdown of FOXP2 (blue) results in a trend toward a decrease in the amplitude of evoked post synaptic potential in LMAN neurons in response to HVC stimulation compared to the control (green) animals ($p = 0.10$, t-test). B) Reduction in the levels of FoxP2 has no effect on the coefficient of variation of synaptic latency from HVC to LMAN ($p = 0.37$, t-test).

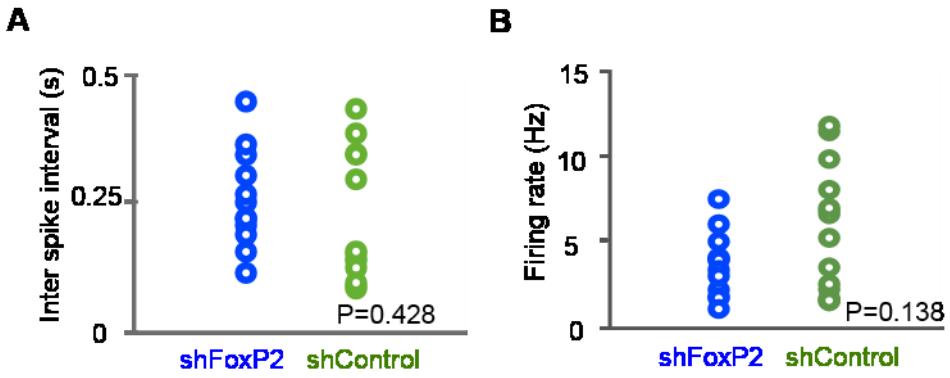

Figure 20: Knockdown of FoxP2 does not affect spontaneous activity of LMAN neurons in the anaesthetized state

A) Reduction in the levels of FoxP2 has no effect on the inter spike interval of LMAN neurons ($p = 0.43$). B) Reduction in the levels of FoxP2 has no effect on the firing rate of LMAN neurons ($p = 0.14$).

4.2.2 Knockdown of FoxP2 renders signal propagation through the AFP insensitive to dopaminergic modulation

A prior study reported that infusing a D1R antagonist into Area X of adult male zebra finches abolishes the reduced syllable variability characteristic of directed singing (Leblois and Perkel, 2010), similar to the vocal effects of injecting LV-ShFoxP2 into Area X of adult birds observed here. These similar behavioral effects, along with the theorized role for dopamine receptor-dependent signaling in modulating singing-related neural activity in the AFP (Doya and Sejnowksi, 1995; Fee and Goldberg, 2011) caused us to wonder whether the altered AFP signal propagation times we observed in the LV-ShFoxP2 animals reflect impaired striatopallidal sensitivity to dopamine signaling. To test this idea, we measured synaptic responses in LMAN neurons evoked by periodic (0.17 Hz) electrical stimulation in HVC before and after we injected a D1R antagonist (SCH) into Area X through a puffer pipette (Figures 21A, see Methods). Application of SCH to Area X of adult male zebra finches significantly and reversibly decreased the mean latency of synaptic responses evoked in LMAN by HVC stimulation, indicating that the timing of activity propagation through the AFP is influenced by dopaminergic signaling in Area X (Figures 21B and C; synaptic latency: baseline = 16.9 ± 0.6 ms, SCH: 15.2 ± 0.6 ms; control: n = 8 cells from 4 adult finches that had not been injected with any LV-constructs; p = 0.03 and 0.02 for 1 and 5 min post drug injection, respectively). In contrast, the latency of HVC-evoked synaptic responses recorded in LMAN neurons of LV-ShFoxP2-injected adult finches was unchanged by SCH application in Area X (Figures 21B and D; synaptic latency

ShFoxP2, baseline: 11.8 ± 0.6 ms, SCH: 12.0 ± 0.3 ms, n = 5 cells from 3 birds; p = 0.7). Similarly, infusing a D1R agonist (SKF) into Area X (Figure 22A) significantly and reversibly increased AFP propagation times in control but not in LV-ShFoxP2 animals (Figures 22B and C, D; synaptic latency in controls: baseline = 15.4 ± 1 ms, SKF = 17.3 ± 1.2 ms, n = 5 cells from 5 birds, p = 0.01 for 1 min post drug injection; ShFoxP2 baseline = 13.1 ± 0.4 ms, SKF = 13 ± 1 ms, n = 5 cells from 3 birds, p = 0.9). In addition, application of SKF or SCH in Area X did not alter the resting membrane potentials or spontaneous firing rates of LMAN neurons in either control or FoxP2 knockdown animals (Table 3). Therefore, D1R-mediated signaling in Area X modulates the speed at which activity propagates from HVC to LMAN, and reducing FoxP2 levels in Area X renders this AFP signal propagation time insensitive to this type of dopaminergic modulation.

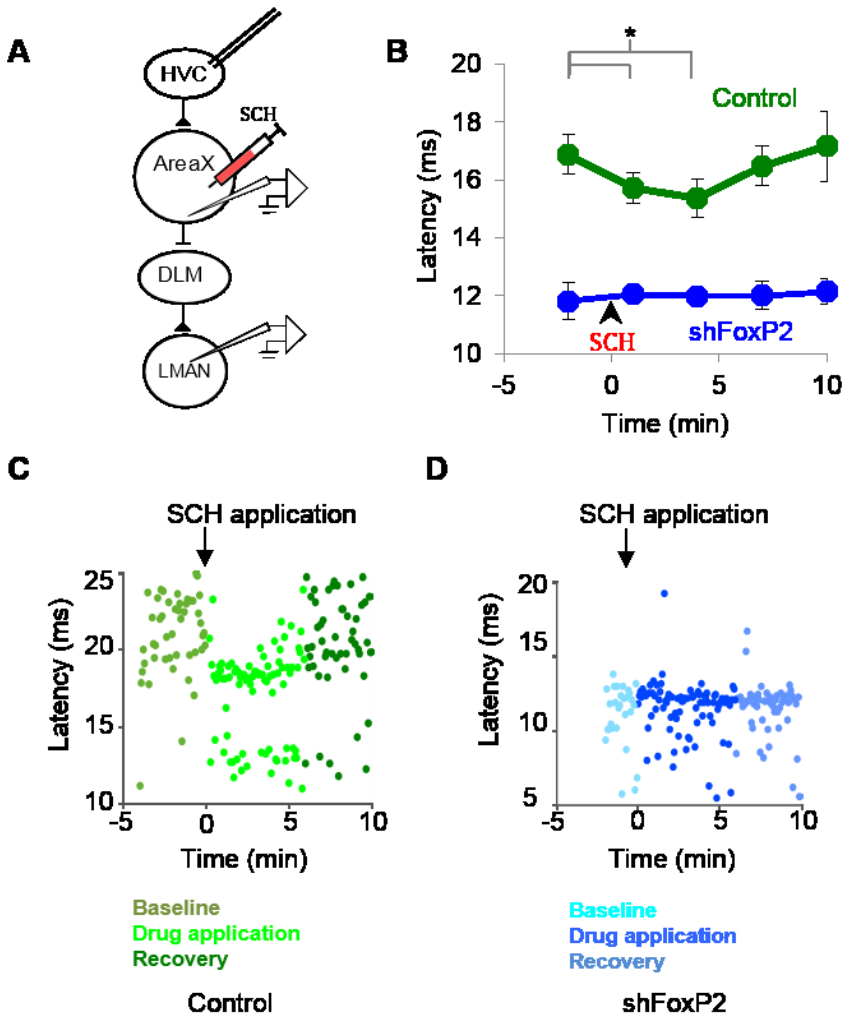

Figure 21: Knockdown of FoxP2 renders signal propagation insensitive to modulation by a dopamine antagonist

A) A schematic depicting the placement of the stimulation and recording electrodes and the puffer pipette. B) In adult male zebra finch controls, the AFP signal propagation time transiently decreases following the injection of D1R antagonist (SCH) into Area X (1 and 4 min post drug delivery, $p < 0.05$, green, $n = 8$ cells, 4 birds). In contrast, the AFP signal propagation time in adult finches previously injected with LV-ShFoxP2 (blue, n = 5 cells, 3 birds) is unaffected by the SCH injection. C-D) Representative cells from a control (left; green) and FOXP2 knockdown (right; blue) animal respectively showing the changes in synaptic latency from HVC to LMAN following the application of a D1R antagonist, SCH (drug application indicated by an arrow, sampling rate: 0.25Hz).

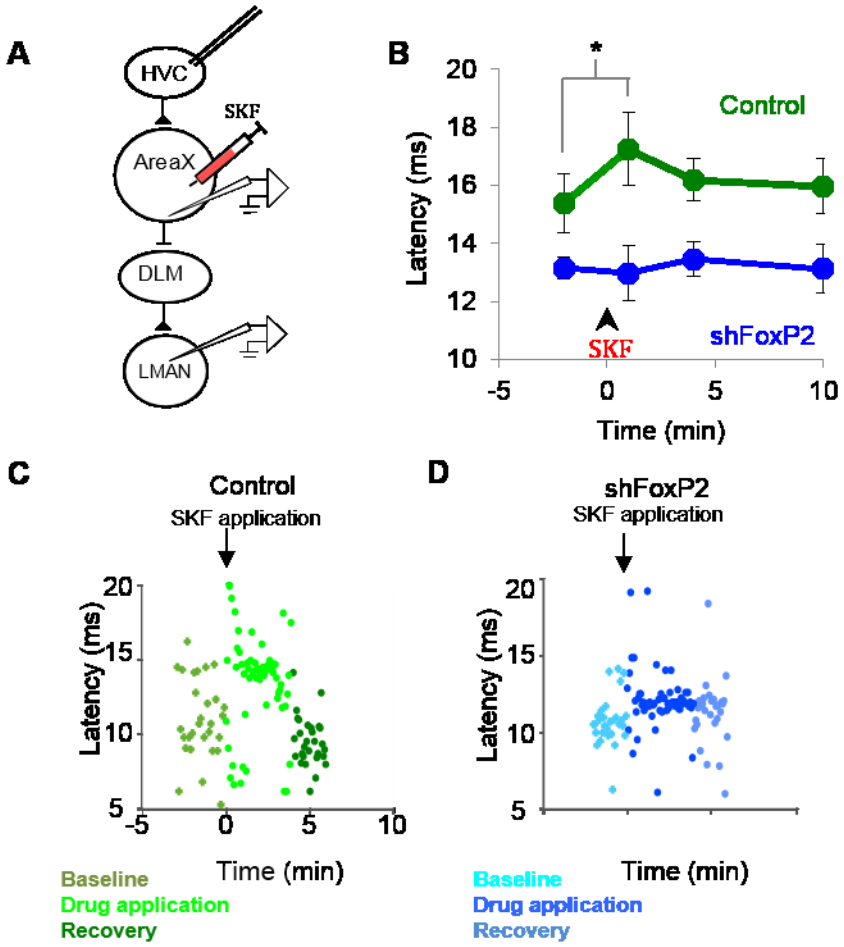

Figure 22: Knockdown of FoxP2 renders signal propagation insensitive to modulation by a dopamine agonist

A) A schematic depicting the placement of the stimulation and recording electrodes and the puffer pipette. B) The AFP signal propagation times measured in control finches transiently increases following the injection of D1R agonist (SKF) into Area X (green, 1 min post drug delivery, $p < 0.05$, n = 5 cells, 5 birds). In contrast, AFP signal propagation times in the FoxP2 knockdown animals (blue, n = 5 cells, 3 birds) are unaffected by SKF injection. C-D) Representative cells from a control (left; green) and knockdown animal (right; blue) respectively showing the changes in synaptic latency from HVC to LMAN following the application of a D1R agonist, SKF.

Table 3: Application of D1R agonist or antagonist has no effect on the resting membrane potentials and spontaneous firing rates of LMAN neurons in both control and FoxP2 knockdown birds

Resting membrane potential (mV)					
Group	Drug	Baseline	Drug	Recovery	P-value
Control	SKF	-70.9 ± 4.05	-71.5 ± 6.26	-73.9 ± 8.61	0.94
ShFoxP2	SKF	-73.4 ± 3.19	-75.5 ± 3.03	-73.1 ± 3.66	0.99
Control	SCH	-73.4 ± 1.31	-72.7 ± 3.61	-69.2 ± 6.33	0.71
ShFoxP2	SCH	-79.4 ± 4.84	-72.9 ± 2.80	-79.3 ± 2.10	0.37
Spontaneous firing rates (Hz)					
Control	SKF	3.88 ± 1.39	2.44 ± 1.59	3.01 ± 2.03	0.59
ShFoxP2	SKF	2.98 ± 1.14	2.55 ± 0.99	3.97 ± 1.68	.61
Control	SCH	3.42 ± 1.25	2.56 ± 1.14	2.93 ± 1.47	0.70
ShFoxP2	SCH	2.38 ± 1.58	2.35 ± 1.54	2.61 ± 2.14	0.59
ANOVA, p values for all conditions and groups were > 0.05					

4.2.3 Millisecond differences in AFP timing can affect spike timing variability in RA.

The AFP exerts its effects on syllable variability through the synapses that LMAN axons make on RA neurons (Olveczky et al., 2005), and the variability of action potential timing in RA neurons can account for a significant fraction of acoustic variations in the bird's song (Sober et al., 2008). These observations along with our present findings advance a model in which spike timing variability in RA is affected by the relative latencies of singing-related activity arriving from LMAN and HVC. To begin to test this model, we used sharp intracellular current clamp recordings in brain slices to measure the timing of action potentials in RA neurons evoked by electrically stimulating LMAN and HVC axons at different latencies (Figure 23A, n = 23 cells from brain slices prepared from 5 adult male zebra finches, 120dph ± 10dph). Specifically, we measured the variability of RA action potential timing (i.e., "spike jitter") when the interval between LMAN and HVC axon stimulation was varied from 0 to ± 5 ms, a range that encompasses the small but significant change we observed in AFP signal propagation times in LV-ShFoxP2 animals (Figure 23B). We found that RA spike timing variability was significantly higher when the interval between LMAN and HVC axon stimulation was lengthened from 0 to ± 3 ms and ± 5 ms (Figures 23C, D; $p < 0.05$). Therefore, millisecond differences in the arrival times of signals from LMAN and HVC are sufficient to affect spike timing variability in RA neurons, providing a plausible mechanism by which

millisecond changes in AFP signal propagation times could affect spectral variability of the bird's song.

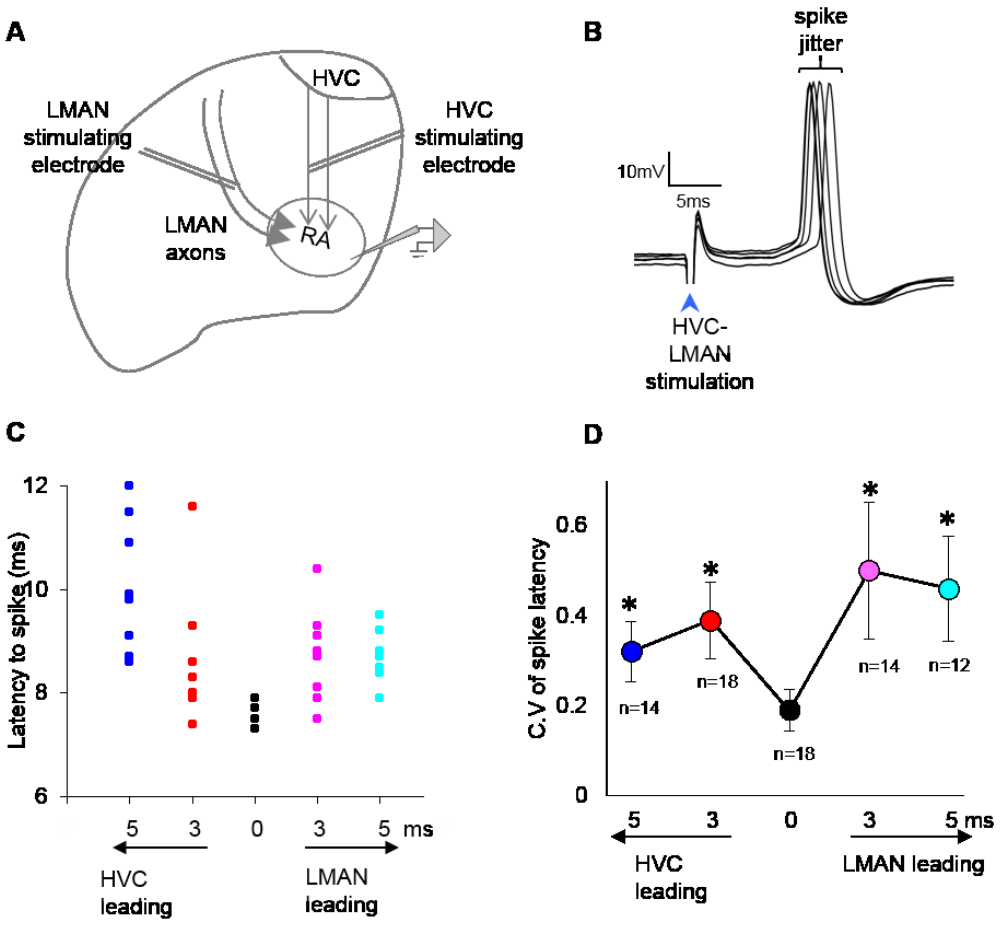

Figure 23: Millisecond differences in HVC and LMAN axon activity affect action potential timing variability in RA

A) A schematic of a coronal brain preparation showing HVC and LMAN axon tracts in relation to RA and the relative placement of the stimulation and recording electrodes. B) Intracellular membrane potential recording from an RA neuron in a coronal brain slice preparation showing action potentials evoked by brief and simultaneous electrical stimulation of HVC and LMAN fiber tracts (HVC- 50 μA, LMAN-100 μA, 100 μs, bipolar electrodes). C) A raster plot of action potentials evoked from a RA neuron by stimulating HVC and LMAN axon tracts at varying relative latencies. Each color represents a different relative stimulation time of the axon tracts. D) The mean coefficient of variation in spike latency is at a minimum when HVC and LMAN axons tracts are simultaneously stimulated and is significantly higher when the absolute relative latencies in stimulation times are increased to 3 and 5 milliseconds (error bars represent S.E.M, $p < 0.05$, n = number of RA neurons that contributed to each measurement).

4.3 Discussion

The goal of this Chapter was to understand how knockdown of FoxP2 in the striatum could generate circuit level defects that could account for behavioral deficits.

First, I used *in vivo* intracellular recordings from LMAN neurons of anesthetized male zebra finches to establish that FoxP2 knockdown in Area X accelerates signal propagation from HVC to LMAN. Combining the above experiment with pharmacological manipulations in Area, I observed that knockdown of FoxP2 renders signal propagation time insensitive to modulation by dopamine receptor 1 (D1R). Furthermore, in collaboration with Stephen Harward, we performed western blot experiments on striatal samples of FoxP2 knockdown animals and we observed that knockdown of FoxP2 results in reduced levels of D1Rs and DARPP32 (see Appendix A). These experiments provide a plausible molecular mechanism by which knockdown of FoxP2 can render signal propagation through the AFP insensitive to D1R-mediated modulation.

Finally, using a novel electrophysiological slice assay I wanted to test if small differences in signal propagation times through the AFP (~ 3 ms) could alter RA spike jitter. Interestingly I found that even a small difference in the relative latencies of HVC and LMAN stimulation times is sufficient to significantly alter RA spike times, a neural correlate of song variability (Sober et al., 2008). Furthermore, accelerated signal propagation through the AFP could be a resultant of increased correlated activity that

have been reported in hypodopaminergic corticostriatal circuits in mice (Costa et al., 2006; Wickens et al., 2007). Heightened correlations in HVC – Area X activity could accelerate signal propagation through the AFP and augment bursting activity in LMAN, either or both of which could contribute to vocal variability.

Taken together, our findings show that knockdown of FoxP2 accelerates signal propagation and renders this signal insensitive to dopaminergic modulation. These signal propagation deficits and impaired D1R-mediated modulation likely contribute to the augmented burst firing of LMAN neurons (Chapter 2) and increased song variability (Chapter 1) observed during directed singing in the FoxP2 knockdown animals.

4.4 Methods

All electrophysiological data were collected inside a sound-attenuating chamber (Industrial Acoustics) placed on an air table (Technical Manufacturing Corporation) and using a data acquisition board (National Instruments) controlled by custom Labview software. Borosilicate glass electrodes (80-150 MΩ, BF100-50-10, Sutter instruments) filled with 2M potassium acetate were used to obtain sharp intracellular recordings of LMAN and RA neurons. Membrane potential recordings were amplified with an Axoclamp 2B amplifier (Axon Instruments) in bridge mode, low-pass filtered at 3 kHz, and digitized at 11 kHz (10 kHz for slice data).

4.4.1 *In vivo* intracellular experiments

A day prior to the *in vivo* intracellular experiment, birds were anaesthetized with 2% isofluorane and a stainless steel post was mounted on the bird's skull with dental cement. On the day of the experiment, the birds were anaesthetized with either 20% urethane (30-40 μL doses every 30 minutes to final volume of 90-120μL) or diazepam (50 μl, 2.5 mg/mL) injected into their pectoral muscles. Urethane was used for all the pharmacological experiments to enable recording for a longer time. The average latencies obtained using both anesthetic agents were comparable (mean latency, ms: ShFoxP2diaz: 12.5 ± 0.3, ShFoxP2ure: 12.4 ± 0.4, shCdiaz: 15.4 ± 0.3, shCure: 16.4 ± 0.7 ms). The body temperature was maintained at ~37°C using a heat blanket (Harvard apparatus). The target nuclei (coordinates: LMAN- 5.3 mm rostral, 1.9 mm lateral, HVC – 0 mm caudal, 2. 4 mm lateral and Area X- 6.3 mm rostral, 1.6 mm lateral or 5.3 mm rostral and .5 mm lateral) were measured from the midsagittal sinus bifurcation and craniotomies (~200 μm by 200μm) were made over the target nuclei (LMAN, HVC and Area X). Target regions were identified by their characteristic activity patterns; in addition, LMAN neurons were identified based on their characteristic spike shape and response to HVC stimulation. Sharp intracellular recordings of LMAN neurons were obtained while HVC was electrically stimulated using bipolar tungsten electrodes (0.1 MΩ, Microprobes) with the tips spaced ~300 μm. Biphasic stimulation for 400 μs was applied at a current strength of 40 μA. Synaptic latency and amplitude of postsynaptic potentials (PSPs) of LMAN

responses to HVC stimulation were measured from median filtered traces using custom event detection software (Matlab, K.Hamaguchi). To quantify 'spikes' for intracellular and extracellular data, the threshold was manually assigned (and kept constant across conditions) to a value above the baseline and events that crossed the threshold were counted as spikes. The mean spontaneous firing rates and inter-spike intervals (ISIs) were measured from recordings of spontaneous activity during a 1s baseline period prior to HVC stimulation. Standardized two-tailed t-tests for independent samples were performed to test for statistical significance between the control and the knockdown groups.

4.4.2 Pharmacology experiments

For the pharmacology experiments, a glass pipette (tip diameter 15–20 μm) was attached to a tungsten electrode (0.5–1.0 MΩ, Microprobes). The glass pipette was filled with either 0.5 mM SKF 38393 hydrobromide (Tocris Bioscience) or 5 mM SCH 23390 (Sigma) in .9% saline and ~30-60 nl was injected using a Picospritzer II (General Valve) in 50–150 ms pulses at 30 psi (Leblois et al., 2010). The tungsten electrode was used to record multiunit neural activity in Area X and electrode placement was confirmed on the basis of robust responses in Area X to HVC stimulation. The glass pipette-tungsten electrode into Area X was lowered at a 25-30° to avoid passing through LMAN. In addition, previous work has shown that LMAN has very low expression levels of D1 receptors (Kubikova et al., 2010). In a subset of experiments a red dye (Alexa Fluor 594 Cadaverine) was added to the drug

to monitor the spread of the dye post hoc. Mean firing rate of Area X was calculated from multiunit recordings using custom written software (Matlab). Control birds used for pharmacological experiments received no viral injections. Sharp intracellular recordings of LMAN neurons with HVC stimulation were performed as described above. Each hemisphere was limited to a single instance of drug delivery. A two-factor ANOVA with repeated measures on one factor was used to test for statistical significance in latency measurements followed by post hoc two-tailed t-tests for correlated samples.

4.4.3 *In vitro* slice experiments

All birds used were older than 90 dph. For the in vitro slice experiments, birds were anaesthetized with isoflurane (5%) and decapitated. The brain was quickly removed and moved into a solution of ice-cold artificial cerebrospinal fluid (aCSF). 400 μm coronal brain slices including RA were cut using a vibratome (Leica, VT 1000s). Concentric tungsten bipolar stimulation electrodes (FHC) were placed ≤ 1mm lateral and dorsal to RA to stimulate either the LMAN or HVC axon tracts respectively (Mooney, 1992). Each axon tract was stimulated independently using A360 Stimulus Isolaters (World Precision Instruments) at 30-200 μA for a duration 100-200 μs while intracellular sharp recordings were obtained from RA neurons. Only cells (23 cells, 5 normal birds) that responded to both HVC and LMAN stimulation were included in the analysis. A subset of the 23 cells contributed to each stimulation parameter, all birds contributed to each parameter, and trial blocks were pseudo randomized to avoid bias. A Master-8 (A.M.P.I) controlled by

custom LABVIEW software was used to allow either HVC or LMAN stimulation to lead the other by 0, 3 or 5ms. Spike latency was determined using custom software (MATLAB).

4.4.4 Statistics

Single or two-factor ANOVA was used to test for statistical significance followed by post-hoc Student t-tests.

5. Conclusions and future directions

Here I combined behavioral assays, genetic manipulations, and *in vivo* intracellular recordings to demonstrate that lentiviral shRNA-mediated knockdown of FoxP2 in Area X increases song variability in both juvenile and adult zebra finches, while also accelerating signal transmission through the AFP. We found that signal propagation times through the AFP were modulated by dopamine receptors of the D1 subtype in adult male zebra finches but that this D1R-dependent modulation was absent following viral-mediated reduction of FoxP2 in Area X. Furthermore, in collaboration with Stephen Harward we found that FoxP2 knockdown decreased levels of D1R and DARPP32, providing a plausible molecular basis for these physiological deficits. Using intracellular recordings in brain slices, I demonstrated that small changes in signal propagation times through the AFP similar to those observed following FoxP2 knockdown in Area X are sufficient to drive increased variability in the action potential timing of RA song premotor neurons, an electrophysiological correlate of song variability (Sober et al., 2008). Finally, in addition to preventing context-dependent changes in song variability in adult birds, FoxP2 knockdown in Area X abolished context-dependent modulation of burst firing in LMAN, the output nucleus of the AFP that drives song variability through its synaptic connections to RA neurons. Taken together, these findings show that FoxP2 knockdown in Area X interferes with the D1R-dependent modulation of activity propagation in the AFP and the context-dependent modulation of singing-related activity in LMAN. These

neuronal deficits disrupt the adult bird's ability to modulate performance variability as a function of social context and could diminish the juvenile's capacity to harness performance variability that is theorized to facilitate song copying.

5.1 Knockdown of FoxP2 in Area X affects song variability in adult and juvenile finches

Since the *FOXP2* gene is expressed in humans since embryonic development onwards, understanding the nature of the 'core' behavioral deficits in humans with *FOXP2* mutations has been difficult. While deficits in articulation, an inability to control fine orofacial movements, and trouble with fluent repetition of word and non-word sequences point to a role for FOXP2 in ongoing motor control, distinguishing the role of FOXP2 in acute speech control from developmentally restricted learning mechanisms remains complicated.

Similarly, while a prior study demonstrated that FoxP2 knockdown affects the ability of juvenile zebra finches to accurately imitate their tutor's song, exactly how learning was affected remained unclear (Haesler et al., 2007). One clue for the role of FoxP2 in the acute control of song variability comes from a study that monitored FoxP2 mRNA levels as adult male zebra finches switched between social contexts. The study reported reduced FoxP2 mRNA levels during directed singing (Teramitsu and White, 2006). However, a context-dependent difference in FoxP2 protein levels was not evident (Miller et al., 2010). Moreover, these studies did not test causality and there are instances in the literature where correlations in gene expression and behavior do not reflect a causal

linkage (Kimpo and Doupe, 1997; Figure 4A- White et al., 2013). Therefore, whether changes in mRNA or protein affect acute song production, particularly the regulation of song variability, was left unanswered. In my study, I used the ability of adult male zebra finches to modulate song variability in a context-dependent manner as an assay to understand the role of FoxP2 in the acute control of song variability.

5.1.1 FoxP2 knockdown abolishes context-dependent variability in adult song.

I found that shRNA-mediated reduction in FoxP2 protein levels in Area X of adult male zebra finches abolished context-dependent differences in song variability. The directed songs produced by FoxP2 knockdown birds remained variable in the presence of a female bird. The implications of these findings are five-fold. First, the finding that decreased FoxP2 levels in Area X affect adult song performance demonstrates a role for FoxP2 in the acute control of song variability and this is first study to make that distinction. Second, it rules out a model where this gene only contributes to juvenile song learning. Third, these findings identify a novel role for the gene. It extends role of FoxP2 to encompass the adult's ability to generate appropriate behavioral responses to salient social cues. The ability of male birds to sing less variable songs in the presence of a female bird is important in eliciting a successful courtship response from female birds. Since female zebra finches (both naïve and mated) prefer the more stereotyped directed song to the variable undirected songs (Woolley and Doupe, 2008), the variable directed songs produced by FoxP2 knockdown birds indicate a failure in social communication.

However, it is important to note FoxP2 knockdown does not affect higher order song features that change with social context, such as tempo, the number of introductory elements in a motif and number of motifs in a bout. Prior studies have shown that these higher order song features are LMAN independent and are not modulated by D1R-mediated signaling (Kao and Brainard, 2006; Leblois and Perkel, 2012). Fourth, the ability to modulate song variability as a function of social context is dependent on dopamine signaling (Sasaki et al., 2006). The infusion of D1R antagonist into the striatum results in increased variability during directed singing, similar to the results observed in FoxP2 knockdown animals (Leblois et al., 2010). This raises the possibility that knockdown of FoxP2 could result in increased song variability by disrupting D1R-mediated signaling in the AFP. Finally, the increased song variability in adult birds with FoxP2 knockdown raises the possibility that a diminished ability to control and modulate song variability could explain how decreased FoxP2 levels in Area X interfere with song copying.

5.1.2 FoxP2 knockdown results in increased variability in juvenile song.

The ability to control and modulate song variability during development is thought to play an important role in reinforcement models of song learning (Doya and Sejnowski, 2000; Fee and Goldberg, 2011). A popular model is that juvenile birds harness song variability to explore a vocal motor space and use auditory feedback to reinforce those renditions that best match the tutor song. While a direct link between acute song variability and song learning remains elusive, it is likely that acute motor exploration will

play a role in vocal imitation. In support of this idea, as sensorimotor learning progresses the amount of song variability and Wiener entropy (a measure of noisiness of a syllable, with a lower value corresponding to less noisy syllable) produced by juvenile birds decrease (syllables become less noisy; Tchernichovski et al., 2001) and the at end of this sensorimotor learning they begin to sing a highly stereotyped crystallized song. Furthermore, lesions to structures in the AFP in juvenile birds that result in increased (Area X lesions) or decreased (LMAN lesions) song variability adversely affect copying of the tutor song (Scharff and Nottebohm, 1991). Therefore, decreased FoxP2 levels in Area X could prevent accurate imitation of a tutor song by interfering with the juvenile bird's ability to efficiently generate and select those motor programs that best match the tutor song model. In order to determine how FoxP2 knockdown affected ongoing song production in juvenile birds, I recorded the songs of juvenile zebra finches over the entire course of song development (45-95 dph) and I observed that juvenile birds that received LV-ShFoxP2 injections in Area X produced syllables that were more variable in pitch and noisier in comparison to those produced by their control-injected counterparts.

Reflecting in part these changes, juveniles with FoxP2 knockdown produce songs that are poorer copies of the tutor song compared to the control animals over the entire course of development (45-95dph). However, despite these marked differences in their abilities to accurately copy a tutor song, LV-ShFoxP2 and LV-ShC injected animals displayed comparable developmental trajectories to their final adult songs. Taken

together, these findings implicate increased song variability in impaired song copying observed in the FoxP2 knockdown birds. Similar deficits in acute control of speech in humans could underlie the behavioral deficits observed in humans with *FOXP2* mutations. Thus, these novel and important findings significantly advance our understanding of the 'core' behavioral deficits associated with reduced FoxP2 levels in humans and songbirds.

5.1.2.1 FoxP2 knockdown likely affects plasticity mechanisms besides increased variability in juvenile song.

It is important to note that these studies do not exclude the possibility that decreased FoxP2 levels may also affect mechanisms besides those that drive trial-by-trial variations in song performance. Indeed there is evidence from the mouse literature that expressing the mutant Foxp2 gene in mice abolishes striatal LTD and Foxp2 KO mice also exhibit deficits in motor skill learning and motor associative learning suggesting a role for FoxP2 in longer term synaptic and behavioral consolidation (Kurt et al., 2010 ; Groszer et al., 2008). In support of this idea, there is evidence that knockdown of FoxP2 in juvenile birds result in syllable omissions and in some instances, differences in syllable sequence (Haesler et al., 2007). Furthermore, it was observed that FoxP2 levels in Area X in a subset of adult birds that had received injections of either LV-ShC or LV-ShFoxP2 early in development (~20dph) correlated with tutor song similarity. In summary, reducing FoxP2 levels in Area X of the juvenile affects acute syllable production while also interfering with song learning over a longer time course.

5.2 Identifying a neural correlate of increased song variability observed in FoxP2 knockdown animals.

The output of the AFP has been implicated in the acute control of song variability. LMAN lesions and reversible inactivation in juvenile birds decrease trial-by-trial variations in plastic song (Scharff and Nottebohm, 1991; Olveczky et al. 2005). Furthermore, LMAN lesions in adult birds abolish context-dependent differences in song variability (Kao and Brainard 2006).

5.2.1 FoxP2 knockdown abolishes context-dependent differences in LMAN activity

Indeed, there is evidence that LMAN neurons actively drive song variability by modulating RA activity through the synapses they make on RA neurons. LMAN neurons exhibit more variable activity and elevated bursting activity when juvenile birds sing more plastic song (Olveczky et al., 2005). Similarly, LMAN neurons in adult birds display increased trial-by-trial variability, higher firing rates and augmented bursting activity during undirected singing relative to directed singing (Kao et al., 2008; Stepanek and Doupe, 2010; Hessler and Doupe 1999b). Furthermore, song-triggered microstimulation of LMAN neurons has acute effects on song (Kao et al., 2005). Taken together, these studies strongly suggest that elevated firing activity and augmented burst firing of LMAN neurons underlie acute changes in song variability. Therefore, it is reasonable to assume that FoxP2 knockdown alters LMAN activity patterns, such that it drives increased song variability during directed song.

In this study I used chronic extracellular recordings to monitor the activity of LMAN neurons in different social contexts. I observed that LMAN neurons recorded in FoxP2 knockdown animals maintained elevated firing rates, higher trial-by-trial variability and augmented bursting activity during directed singing, thereby abolishing context-dependent differences in LMAN activity. Furthermore, these changes in LMAN activity were accompanied by a loss in context-dependent modulation of song variability. It was observed that firing rates, trial-by-trial variability and bursting activity of LMAN neurons during directed singing in the FoxP2 knockdown animals were substantially higher compared to their control counterparts. Based on prior findings it is likely that these changes in LMAN activity are translated into more variable spiking in RA that is correlated with, and thought to drive greater spectral variability in the bird's song (Olveczky et al., 2011; Sober et al., 2008).

Furthermore, these changes in LMAN activity were reflected in the comparisons of the ISI distributions of the LMAN neurons in the control and knockdown animals. Interestingly, while no changes were observed in the amount of song variability during undirected singing in the FoxP2 knockdown and control birds, subtle differences in the ISI distributions of LMAN neurons were observed. The ISI distribution of the of LMAN neurons in FoxP2 knockdown animals were left shifted compared to the control animals, particularly in the time period (2-5ms) that corresponds to bursting activity in LMAN neurons. This finding raises the possibility that while differences in the amount of song

variability in the FoxP2 knockdown and control animals in the directed context are obvious, there might also be subtle effects on the variability of undirected songs that might be difficult to detect from a noisy baseline.

Unlike the elevated firing rates and augmented bursting activity observed in LMAN neurons of FoxP2 knockdown animals during directed singing, lesions to Area X abolish bursting activity of LMAN neurons while having no effects on their singing-related increase in firing rate (Kojima et al., 2013). This rules out a scenario in which the behavioral and circuit deficits observed simply arise as a result of LV-ShFoxP2 injections merely lesioning Area X by causing infected MSNs to die. Furthermore, no differences were observed in the actin levels in Area X tissue samples from both FoxP2 knockdown and control animals showing that injections of LV-ShFoxP2 does not lesion Area X.

It is likely that FoxP2 knockdown in juvenile birds has similar effects on the activity of LMAN neurons as in the adult animals, thereby resulting in songs that are more variable and noisier compared to control animals and ultimately impairing song copying in these birds. It will be interesting to test in future experiments in juvenile FoxP2 knockdown birds if this indeed is the case. In summary, FoxP2 knockdown in Area X of adult birds result in elevated firing rates, increased trial-by-trial variability and augmented bursting activity during directed singing that likely drives more variable spiking in RA, that in turn could result in increased song variability during directed singing, and ultimately abolishing context-dependent differences in song variability. Thus

in this study we have identified changes in LMAN activity as a plausible neural correlate of increased song variability observed in FoxP2 knockdown animals.

5.3 FoxP2 knockdown reduces levels of D1Rs and DARPP-32, key molecules involved in D1R-mediated signaling

A major limiting factor in identifying key molecular mechanisms that underlie deficits in circuit function and behavior associated with FoxP2 knockdown is that transcription factors such as FoxP2 can alter the expression of hundreds or even thousands of downstream genes and thus have the potential to affect brain function and behavior through a wide variety of mechanisms (Hilliard et al., 2012; Vernes et al., 2011). Nonetheless, several observations implicate FoxP2-mediated regulation of dopaminergic signaling in the learning and performance deficits that have been measured in FoxP2 knockdown zebra finches (Haesler et al., 2007; current findings). First, dopamine levels in Area X increase during directed singing and infusion of a D1R antagonist into Area X can abolish context-dependent changes in song variability, similar to the effects of FoxP2 knockdown observed in this study (Sasaski et al., 2006; Leblois et al., 2010). Second, several genes implicated in neuromodulatory signaling pathways, including a gene that encodes an enzyme that metabolizes dopamine (monoamine oxidase B, MAOB), are differentially regulated by FOXP2 in humans versus chimps (i.e., FOXP2chimp) (Konopka et al., 2009; Spiteri et al., 2007; Vernes et al., 2007). Furthermore, gene expression studies have shown that FoxP2 regulates several molecules implicated in D1R-mediated signaling, including DARPP-32, which is co-expressed with FoxP2 in D1R expressing

MSNs in both zebra finches and mice (Vernes et al., 2011; Haesler et al., 2004; Heiman et al., 2008; Konopka et al., 2009). Finally, dopaminergic signaling has been advanced as a key player in reinforcement learning, which is theorized to contribute to song motor learning (Reynolds et al., 2001; Doya and Sejnowski, 1995; Fee and Goldberg, 2011).

Indeed, it was observed that knockdown of FoxP2 in Area X significantly decreased levels of DARPP-32 and D1Rs (Appendix A). While D1Rs are not thought to be direct targets of FoxP2 regulation, D1Rs have been shown to be concentrated in dendritic spines of medium spiny neurons (MSNs; Levey et al., 1993 and Yao et al., 2006) and knockdown of FoxP2 leads to a decrease in MSN spine density in songbirds (Schulz et al. 2010). In addition, I found that FoxP2 knockdown in Area X renders signal propagation through the AFP insensitive to D1R-mediated signaling. Taken together, these findings support a model where FoxP2 is necessary for D1R-mediated control of striatal signaling important to effective communication in adults and efficient learning-related vocal motor exploration in juveniles.

5.4 Signal propagation deficits in the AFP and song variability

Disrupted FoxP2 expression can affect the structure and function of striatal MSNs in both mice and songbirds (Schulz et al., 2010; Enard et al., 2009; Groszer et al., 2008). Moreover, mice with FoxP2 mutations are impaired in motor skill learning and auditory-motor associative learning (Enard et al., 2009; Groszer et al., 2008; Kurt et al., 2010) and also display patterns of striatal activity that are abnormally high and aberrantly

modulated during motor skill learning (French et al., 2012). Nonetheless, how these striatal deficits affect the function of circuits important to vocal performance and learning has remained unclear, in part because mice vocalizations appear to be innate (Kikusui et al., 2011; Hammerschmidt et al., 2012; Mahrt et al., 2013)

5.4.1 Knockdown of FoxP2 accelerates signal propagation through the AFP

By performing electrophysiological recordings in LMAN while stimulating HVC following FoxP2 knockdown in Area X, I observed that signal propagation times through the AFP were faster in the FoxP2 knockdown animals compared to the control animals. Furthermore, no changes were observed in other signal propagation parameters such as the amplitude of the synaptic responses and the coefficient of variation in synaptic latency.

5.4.2 FoxP2 knockdown renders signal propagation times insensitive to D1R-mediated modulation

The findings from the current study and several observations from prior studies (see section 5.3) implicate deficits in dopaminergic signaling in the accelerated signal propagation times observed in the FoxP2 knockdown animals. The influx of a D1R antagonist into Area X of an adult male zebra finch has the same effect as the FoxP2 knockdown on the ability of these birds to modulate their song in response to social context (Leblois et al., 2010). Furthermore, it is known that signal propagation through the AFP is sensitive to D1R-mediated signaling (Leblois et al., 2010). To test if the accelerated

signal propagation times observed in the FoxP2 knockdown animals were a result of deficits in dopaminergic signaling, I used intracellular sharp recordings of LMAN neurons and combined them with pharmacological manipulations of Area X activity while stimulating HVC.

In the control animals infusion of a D1R agonist into Area X transiently increased signal propagation times while infusion of an antagonist resulted in decreased them, similar to faster signal propagation times observed in the FoxP2 knockdown animals. However, in the FoxP2 knockdown animals AFP signal propagation times were insensitive to the application of both the D1R agonist and antagonist and remained faster compared to the control animals. These findings suggest that deficits in dopaminergic signaling could underlie the accelerated signal propagation times observed in FoxP2 knockdown animals.

5.4.3 Potential mechanisms that could account for accelerated signal propagation times through the AFP

Notably, these FoxP2- and D1R-dependent changes in AFP propagation times were on the millisecond timescale, raising the question as to how such fine timing differences arise. One possible explanation for accelerated AFP signal propagation following FoxP2 knockdown is that diminished signaling through the D1R+ pathway unmasks or accentuates other pathways. One such pathway is the cortico-thalamocortical loop (HVC-RA-DLM-LMAN) that is also capable of driving LMAN activity (Goldberg and Fee, 2012; Wild, 1993; Vates et al., 1997). DLM neurons are strongly modulated by

singing even in the absence of input from Area X (reversible inactivation or lesion) suggesting that this pathway is capable of driving LMAN neurons. These findings raise the possibility that FoxP2 knockdown reduces the inhibition that Area X pallidal neurons exerts on DLM, therefore disinhibiting DLM neurons that could in turn increase the influence that this pathway exerts on LMAN activity. Since this alternate pathway involves gluatamergic synapses, it can lead to faster signal propagation times observed in the FoxP2 knockdown animals. Furthermore, disinhibition of DLM neurons can result in elevated firing rates and burst firing of LMAN observed in FoxP2 knockdown animals.

However, if accelerated signal propagation times through the AFP observed in FoxP2 knockdown animals were a result of increased influence of the HVC-RA-DLM loop on LMAN activity, it would be reflected by changes in spontaneous firing rates and the EPSP amplitudes of LMAN neurons in response to HVC stimulation. But these parameters remained unaffected in the FoxP2 knockdown animals. Furthermore, there is preliminary evidence (Appendix B) that lesioning most of RA had no effect on AFP signal propagation times in both control and knockdown animals. Signal propagation times through the AFP in RA lesion - FoxP2 knockdown birds remained unaffected by application of a D1R agonist considerably and remained faster compared to their control counter parts with RA lesion. Because these lesions were without any effect, but were also not quite large enough to encompass all of RA, these experiments do not completely rule out the possibility that HVC-RA-DLM-LMAN pathway could play a role in the

accelerated signal propagation times observed in the FoxP2 knockdown animals. Taken together, it appears likely that mechanisms besides altered influence of the HVC-RA-DLM underlie the accelerated AFP signal propagation times observed in the FoxP2 knockdown birds. Another and more likely possibility is that diminished signaling through D1R+ pathways unmasks or accentuates faster propagation through a parallel channel through Area X. Other candidate pathways could include D2R+ MSNs or one involving a direct connection between HVC axons and pallidal cells in Area X (Ding and Perkel 2002; Farries et al., 2005). Indeed, this latter pathway presumably is more direct and thus potentially faster than one involving an additional inhibitory synapse mediated through MSNs. Therefore, any or all of these pathways could contribute to the accelerated AFP signal propagation times observed in FoxP2 knockdown birds and identifying these circuit mechanisms that give rise to accelerated signal propagation times would be an important avenue for future research.

5.4.4 Millisecond differences in AFP timing can affect spike timing variability in RA.

Another remaining issue is whether and how small timing differences in AFP signal propagation times following FoxP2 KD are linked to increases in song variability. One potential clue is that birdsong and the neural mechanisms that give rise to its generation display remarkable temporal precision. During singing, RA neurons produce bursts of action potentials with sub-millisecond precision and remarkably low trial-by-trial variability (Chi and Margoliash, 2001), and small variations in RA action potential

timing can account for a significant portion of song variability (Sober et al., 2008). Furthermore, during singing, HVC transmits both a precisely timed premotor signal that drives RA neuron firing and a precisely timed copy of this signal to the AFP, the output of which (LMAN) can modulate RA action potential variability by activating postsynaptic NMDA receptors on RA neurons (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007; Prather et al., 2008; Long and Fee, 2008; Long et al., 2010; Olveczky et al., 2005; Olveczky et al., 2011; Mooney and Konishi, 1991).

One possibility is that subtle differences in signal propagation times through the AFP could affect the extent to which functional interactions between HVC and LMAN synapses alleviate voltage-dependent blockade of NMDA receptors on RA neurons and thus modulate RA action potential variability during singing. Ultimately, small decreases in AFP signal propagation times, including those resulting from altered D1R amounts induced by FoxP2 knockdown, could drive the system into a persistent state of high variability, whereas activation of D1R-mediated signaling in Area X could normally function to slightly increase AFP signal propagation times and drive the system towards lower variability. In support of this idea, in slice experiments RA spike timing variability was significantly higher when the interval between LMAN and HVC axon stimulation was lengthened from 0 to ± 3 ms and ± 5 ms, a range that encompasses the small but significant change we observed in AFP signal propagation times in LV-ShFoxP2 animals.

Thus providing a plausible biophysical mechanism by which mismatched inputs to RA can generate song variability.

However, the connection between altered AFP signal propagation times and song variability remains untested and an alternative view is that faster AFP signal propagation times following FoxP2 knockdown in Area X reflect other pathological processes that interfere with the modulation of vocal variability. Interestingly, extracellular dopamine concentrations in Area X decrease and singing-related bursting activity in LMAN increases when male zebra finches sing more variable undirected songs (Sasaki et al., 2006; Kao et al., 2008). Indeed, augmented bursting activity in LMAN acts through NMDA receptor-enriched synapses that LMAN axons make in RA to drive more variable spike timing in RA neurons, resulting in greater song variability (Kao et al., 2008; Mooney and Konishi, 1991). Notably, we found that FoxP2 knockdown in Area X renders the striatopallidal circuit insensitive to signaling through D1Rs, while abolishing context-dependent changes in LMAN activity and song variability. These effects of FoxP2 knockdown are suggestive of dopamine hypofunction, which in rodents is associated with an increase in correlated corticostriatal activity (Costa et al., 2006; Wickens et al., 2007). Therefore, the diminished sensitivity to dopamine signaling following FoxP2 knockdown in Area X could trigger pathological levels of correlated corticostriatal (i.e., HVC – Area X) activity during singing. One effect of this increased correlation could be to strongly synchronize inhibitory synapses that pallidal neurons in Area X make on thalamic

neurons that innervate LMAN. Such strongly synchronized inhibition could accelerate signal propagation and facilitate bursting activity in the thalamic-LMAN pathway, perhaps by strongly de-inactivating calcium currents on these thalamic neurons. Ultimately, heightened correlations in HVC – Area X activity could accelerate signal propagation through the AFP and augment bursting activity in LMAN, either or both of which could contribute to vocal variability.

5.4.5 How LMAN bursting activity affects RA spike timing variability

A more fundamental issue is how LMAN bursting, or mismatched timing of HVC and LMAN activity, could affect the variability of RA spike timing. The biophysical evidence points to a critical role for NMDA receptors at LMAN – RA synapses: pharmacological blockade of these receptors is sufficient to strongly reduce song variability in juvenile zebra finches (Ölveczky et al., 2005). Presumably, burst firing of LMAN neurons is more likely to activate NMDA receptors on RA neurons and to alleviate voltage-dependent blockade at these synapses. Furthermore, given the stochastic nature of NMDA receptor-mediated synaptic currents, RA spike timing could become more variable even if LMAN bursting activity was itself temporally invariant. In light of what is known about feedforward inhibitory circuitry in RA (Mooney, 1992), it is also reasonable to assume that the precise timing of LMAN and HVC inputs could determine the extent to which the voltage-dependent blockade of NMDA receptors at LMAN – RA synapses is alleviated. More specifically, feedforward inhibition driven by HVC inputs

to RA could create a transient window in time when voltage-dependent blockade of NMDA receptor mediated currents in RA neurons is accentuated. Conversely, those timing differences in HVC and LMAN activity that permit NMDA receptor activity to fall outside this window could enhance an NMDA receptor mediated contribution to the synaptic currents that flow into RA neurons and thus alter the variability of their spike timing. Although the underlying biophysical and circuit mechanisms await elucidation,

The knockdown of FoxP2 in Area X increases LMAN bursting activity and song variability, in direct contrast to the effects of Area X lesions in adult zebra finches, suggesting that FOXP2 mutations may exert a gain of function effect on brain and behavior (Kojima et al., 2013).

5.5 Future directions

This study has demonstrated an important role for FoxP2 in the acute control of vocal variability and has identified plausible molecular mechanisms and circuit deficits that could underlie the increased song variability observed with FoxP2 knockdown in Area X of songbirds. These findings open several interesting and important directions for future research. A few of these exciting future directions are discussed in the sections below.

5.5.1 Songbird FoxP2 knockdown findings could help inform experiments in Foxp2 KO mice

While mice vocalizations are innate (Kikusui et al., 2011; Hammerschmidt et al., 2012; Mahrt et al., 2013), because of their genetic tractability mice could be an attractive

model system to determine how *FOXP2* mutations affect molecular pathways and circuits important in the acute control of motor variability. Furthermore, Foxp2 KO mice display deficits in motor skill learning and motor-associative learning. While initial studies have reported no changes in motor performance, the parameters used in these studies have been gross measures (Enard et al., 2009; Grozer et al., 2008) and it is possible that Foxp2 KO in mice has subtle effects on ongoing motor performance as has been observed in this study. A more careful analysis of microstructures of limb and head movements in Foxp2 KO mice could help elucidate the role of Foxp2 in the acute control of motor performance in mice. An additional confound is that all published mice behavioral studies have involved reducing Foxp2 levels from embryonic development onwards, thus making it hard to distinguish the role of FoxP2 in the acute control of motor from developmentally restricted learning mechanisms. Therefore, future studies must involve conditional knockout experiments in which Foxp2 levels are reduced in adult mice, an approach that I was able to successfully use in this study using shRNA silencing methods in adult songbirds.

Furthermore, in my thesis I have identified deficits in dopaminergic signaling as plausible a mechanism by which reduced levels of FOXP2 could affect striatal circuit functions and ultimately behavior. These various findings raise the possibility that similar deficits in dopaminergic signaling could underlie behavioral deficits observed in Foxp2 KO mice. Furthermore, mouse models could be useful in identifying molecular

mechanisms and pathways affected by *FOXP2* mutations. In addition to chromatin immunoprecipitation (CHIP) assays and microarray data from mice that implicate dopaminergic signaling molecules as downstream targets of Foxp2 (Vernes et al., 2011), there is evidence that Foxp2 KO mice have increased dopamine levels in the striatum, perhaps suggestive of a homeostatic increase in dopamine levels in compensation to decreased D1R-mediated signaling (Grozer et al., 2008). However, it is important to note that in these experiments the Foxp2 KO was not limited to the striatum and spanned throughout the entire organism, including the midbrain dopaminergic neurons. Thus, the increased dopamine levels could reflect an unrelated mechanism or process. Taken together, the findings from this study could be useful in interpreting existing literature from Foxp2 KO mice and in designing future experiments.

5.5.2 Performing region specific FoxP2 deletions in other brain regions affected by FOXP2 mutations

A major caveat of the current study is that, unlike human subjects with FOXP2 mutations, the knockdown of FoxP2 in birds is limited to the striatum alone. In human patients with *FOXP2* mutations, it is likely that the behavioral deficits observed is a result of conglomeration of deficits spanning multiple brain regions (including but not limited to the striatum). However, as discussed earlier in the introduction (see Section 1.2.1) it appears that striatal abnormalities appear to play an important role in the speech deficits observed with *FOXP2* mutations. The most compelling argument in support of this idea comes from a study by Haesler et al., 2007 and the current study showing that knockdown

of FoxP2 in Area X, a striatopallidal structure is sufficient in disrupting vocal learning and performance. Furthermore, ongoing studies in mice have reported differential behavioral effects with region specific Foxp2 deletions. Foxp2 deletions in the striatum and the cerebellum affects fine microstructures of movements during motor skill learning, while cortical Foxp2 deletions produced no behavioral phenotype (French et al Abstract# 57.09/J9 SFN 2012). These various findings underscore the need for manipulations of FoxP2 that are targeted to specific brain regions, especially those that are known to be important to motor and vocal learning. Therefore, futures studies in mice and birds should focus on reducing FoxP2 levels in specific brain regions and observe their effects on motor/vocal learning and performance. Such targeted analysis would be impossible in human subjects, but could help elucidate the contribution of specific brain regions in the speech deficits observed in humans with *FOXP2* mutations.

5.5.3 Determining the effect of FoxP2 knockdown on the intrinsic and synaptic properties of medium spiny neurons

The FoxP2 protein is highly expressed in the MSNs of birds, mice and humans and reducing levels of FoxP2 is known to affect the structure and function of MSNs (Schulz et al. 2010; Enard et al., 2009; Groszer et al., 2008). Furthermore, FoxP2 KO abolishes long-term depression in the MSNs, a neural substrate for motor skill learning (Groszer et al., 2008). In addition, MSNs in dorsal striatum of Foxp2 KO mice exhibit increased firing rates and aberrant modulation of activity during motor skill learning (French et al., 2012). Since the MSNs are integrators of gluatamergic and dopaminergic signaling, it will be

important to address in future studies how FoxP2 knockdown affects intrinsic and synaptic properties of MSNs. For example, visualized whole cell recordings in brain slices in combination with pharmacological manipulations and Chr2 assisted mapping techniques from LV-ShFoxP2-GFP infected MSNs in Area X of FoxP2 knockdown birds could inform how reduced levels of FoxP2 affect dopaminergic signaling in an individual MSN and how these effects are translated into accelerated AFP signal propagation times observed in FoxP2 knockdown birds. Future experiments should also assess how FoxP2 knockdown alters singing-related activity of MSNs both during song learning in juveniles and context-dependent singing in adults. It will be particularly useful to systematically march through the AFP to assess how FoxP2 in knockdown in Area X is translated into lack of context-dependent changes in LMAN activity and ultimately song variability.

5.5.4 Rescuing FoxP2 knockdown with D1R and/or DARPP-32 overexpression

A major limiting factor understanding the role of FoxP2 in speech learning and control is that transcription factors like FoxP2 can modulate hundreds if not thousands of downstream target genes (Hilliard et al., 2012; Vernes et al., 2011), therefore identifying key molecular pathways and mechanisms underlie behavioral deficits associated with FOXP2 mutations can be difficult. Findings from the current study suggest dopaminergic deficits to play a major role in the song deficits observed with *FOXP2* mutations. An important future direction would be test if song and circuit deficits observed in FoxP2

knockdown birds can be rescued by the overexpression of DRD1 (D1R) and/or Ppp1r1b (DARPP-32) genes in Area X.

5.6 Comparing songbird and mammalian basal ganglia circuits and implications for interpreting findings from this study

While the songbirds and mammalian corticostriatal circuits share several anatomical and functional similarities (Reiner, 2002), including the presence of MSNs in Area X that receives gluatamergic input from HVC and LMAN (equivalent of cortical structures) and dopaminergic input from VTA, they differ in a few ways (Gale and Perkel, 2010). To highlight some of these differences, first, unlike the human and mammalian striatum, MSNs and pallidal-like neurons (pallidal-like neurons appear to be of striatal embryonic origin; Carrilo and Doupe, 2004) in Area X are spatially mixed. Second, in contrast to the mammalian striatum, there is no clear segregation of the D1R and D2R expressing neurons, with nearly half the MSNs in Area X expressing both D1R and D2Rs (Ding and Perkel, 2002; Kubikova et al., 2009). Third, there is no clear anatomical or physiological evidence for the presence of an indirect pathway in the songbird striatum. Fourth, the mammalian basal ganglia sends direct projections to midbrain dopaminergic neurons (VTA and SNc), that shape the activity of these neurons. While no such direct connections have been observed in zebra finches, studies have identified an indirect projection from Area X to VTA via the ventral pallidum (Gale et al., 2008). Keeping these differences in mind, it would be important to test if striatal abnormalities observed in Foxp2 knockout mice such as increased firing rates observed in MSNs and aberrant

modulation of MSN activity during motor tasks (French et al., 2012) are observed in the MSNs in the songbird Area X. Furthermore, it will be important to establish if FoxP2 knockdown in Area X abolishes LTD (Grozer et al., 2008) in MSNs like observed in Foxp2 KO mice. Also, since a large subset of MSNs express both D1Rs and D2Rs, the effect of FoxP2 knockdown might have different effects on intrinsic and synaptic properties of individual MSNs in Area X compared to the effects on D1R MSNs in Foxp2 KO mice.

Despite these differences in the organization of songbird basal ganglia circuitry it appears that many anatomical and functional similarities to mammalian basal ganglia circuits exist, including the fact that FoxP2 knockdown in Area X of zebra finches affects song learning (Haesler et al., 2007) and production (current study) comparable to speech deficits observed in humans with *FOXP2* mutations. Therefore, while it would be important take into consideration the differences between songbird and mammalian basal ganglia circuits, it is likely that the molecular mechanisms and circuit dysfunction that underlie song and speech deficits associated with reduced FoxP2 levels will share many similarities.

5.7 Significance for the role of FOXP2 mutations in human speech deficits

In humans, loss of function mutations in FOXP2 impair speech learning, result in an orofacial dyspraxia, and reduce the volume of the head of the caudate nucleus, a region important to the control of orofacial movements (Hurst et al., 1990; Watkins et al., 2002a; Vargha-Khadem et al., 2005; Watkins et al., 2002b; Belton et al., 2003; Jernigan et al., 1991).

In particular, patients with FOXP2 mutations display severe deficits in articulation (Hurst et al., 1990) and trouble with fluent repetition of word and non-word sequences (Watkins et al., 2002a). While *FOXP2* expression in humans is not limited to the brain, the behavioral deficits in humans with *FOXP2* mutations appear to be limited to brain function, more specifically to deficits in speech and the fine control of orofacial movements. So why do *FOXP2* mutations not affect the development of other organs such as lungs and hearts or exert more general effects on motor control including limb praxis? One possibility is that this reflects a dosage effect and that the unaffected copy of the *FOXP2* gene in these subjects is sufficient for the normal development of the lung, heart and certain regions of the brain (Marcus and Fisher, 2003). An alternate possibility is that other *FOX* genes that share remarkable homology with *FOXP2* and have similar expression profiles (Teramitsu et al., 2004), such as *FOXP1* can compensate for the loss of functional FOXP2 in certain organs and brain areas but not others.

Although birdsong and speech evolved independently, the extreme temporal demands of auditory-motor integration necessary to these learned vocal behaviors may require that striatal pathways operate at their performance limits, with the consequence that songbirds with diminished FoxP2 levels in Area X produce vocal deficits that resemble core aspects of those observed in humans with FOXP2 mutations. Therefore, while FOXP2 may regulate many processes in the human brain necessary to normal language development, one way that FOXP2 mutations could interfere with speech

learning is by disrupting dopaminergic signaling important to the control of vocal variability.

5.8 Summary

In this study I addressed how FoxP2 knockdown in songbird striatum affects vocal control and signal propagation through circuits important for the control of learned vocalizations. In summary, I found that FoxP2 knockdown in the songbird striatum disrupts developmental and social context-dependent modulation of song variability. Recordings in anaesthetized birds show that FoxP2 knockdown interferes with D1R-dependent modulation of activity propagation in a corticostriatal pathway important to song variability, an effect that may be partly attributable to reduced D1R and DARPP-32 protein levels. Furthermore, recordings in singing birds reveal that FoxP2 knockdown prevents social context-dependent modulation of LMAN activity in this pathway. These findings show that reduced FoxP2 levels interfere with the dopaminergic modulation of vocal variability, which may impede song and speech development by disrupting reinforcement learning mechanisms.

Appendix A: Impaired D1-R mediated signaling in Area X

The electrophysiological findings in this thesis raise the possibility that FoxP2 knockdown in Area X interferes with dopaminergic signaling by reducing levels of D1Rs and/or downstream molecules that play a critical role in signal transduction through this receptor. This idea gains support from prior studies showing that FoxP2 knockdown reduces the density of dendritic spines on medium spiny neurons (MSNs), and many D1Rs are localized to MSN spines (Schulz et al., 2010; Levey et al., 1993; Yao et al., 2008). Furthermore, DARPP-32, a key component of the D1R signaling cascade, is a downstream target of FoxP2 (Vernes et al., 2011).

The experiments described below were performed in collaboration with Stephen Harward. To test whether knockdown of FoxP2 affected levels of D1Rs and DARPP-32, we performed western blots of Area X tissue homogenates obtained from animals that had previously received injections of LV-ShFoxP2 or LV-ShC (see Methods). Optical density measurements revealed that FoxP2 knockdown resulted in significantly lower levels of D1R and DARPP-32 levels (Figure 24A-C; D1Rs were reduced 16.6 ± 4.3% and DARPP-32 levels were reduced 88.4 ± 2.8%; $p < 0.05$). Therefore, FoxP2 knockdown reduces levels of D1R and a key molecule that links D1R receptor activation to intracellular signaling cascades, thus providing a probable molecular basis for the physiological and behavioral deficits we observed.

Methods

All birds were placed in a dark chamber without light for 2 hours (~ noon). The birds had received injections of either LV-ShFoxP2 or LV-ShControl ~ 90 days prior to retrieval of brain samples. The birds were anesthetized with 5% isoflurane and decapitated. Their brains were quickly removed from the skull and were mounted on a vibratome (Leica, VT 1000s) stage. 100 μm sections were cut until Area X was visible (~ 1200 μm from the rostral tip of the brain; Miller et al., 2008). GFP fluorescence from the viral infection was used to confirm the location of Area X, there is a possibility that this may have biased us towards sampling from regions of higher labeling density. A 0.8mm tissue punch was used to remove much of the tissue encompassing Area X. Only samples from the left hemisphere were used for the western blot experiments. The samples were quickly frozen in liquid N2 and stored at -80° C until later use. Frozen tissue was homogenized in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate), centrifuged at 16000g for 10 min, and supernatant collected. This supernatant was defined as tissue homogenate and was resolved with SDS-Page. The blots were incubated with FoxP2 (1:500, Sigma-Aldrich), D1R (1:1000, Abcam), DARPP-32 (1:1000, Abcam) and actin (1:10000, Sigma-Aldrich) primary antibodies for at least 12 hours at 4°C followed by incubation with secondary antibodies (1:5000, Jackson Labs) for at least 1 hour at room temperature. Only samples from the left hemisphere were used for the western blot experiments. The blots were incubated with FoxP2 (1:500, Sigma-

Aldrich), D1R (1:1000, Abcam), DARPP-32 (1:1000, Abcam) and actin (1:10000, Sigma-Aldrich) primary antibodies for at least 12 hours at 4°C followed by incubation with secondary antibodies (1:5000, Jackson Labs) for at least 1 hour at room temperature. For quantitative analysis, immunoblots were scanned with a digital scanner and the optical band density was quantified using ImageJ analysis software. Optical densities for FoxP2, D1R, and DARPP-32 were normalized to corresponding actin levels. Data are presented as mean ± SEM. Statistical significance was assessed with the Student's t-test.

Conclusions:

FoxP2 knockdown reduces levels of D1R and DARPP-32, thus providing a plausible molecular basis for the physiological and behavioral deficits we observe with FoxP2 knockdown.

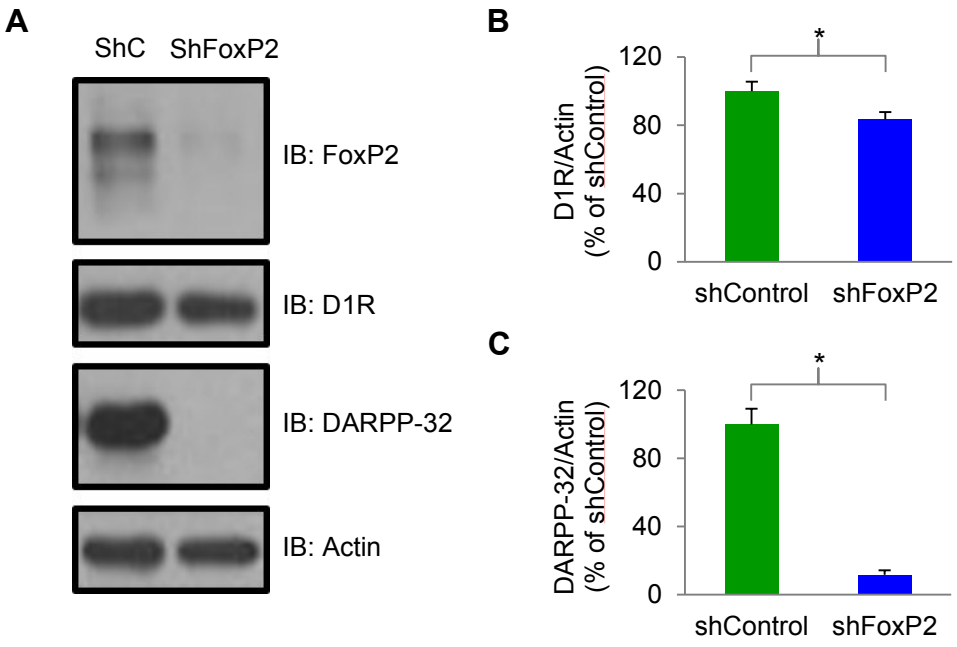

Figure 24: Knockdown of FoxP2 reduces levels of D1Rs and DARPP-32 in Area X

A) A representative immunoblot (IB) showing FoxP2, D1R, DARPP-32 and actin protein levels in tissue punches from Area X of LV-ShC and LV-ShFoxP2 injected birds (>90 days after the injection). B) The Area X tissue samples from the LV-ShFoxP2-GFP injected animals had significantly lower levels of D1Rs compared to the control animals (mean 16% reduction; LV-ShFoxP2: n=11 birds; LV-ShC: n = 11; $p < 0.05$; DR1 levels were normalized to actin). C) The Area X tissue samples from the LV-ShFoxP2-GFP injected animals had significantly lower levels of DARPP-32 compared to the control animals (mean 88% reduction; LV-ShFoxP2: n=11 birds; LV-ShC: n = 10; $p < 0.05$; normalized to actin).

Appendix B: RA lesions have no effect on AFP signal propagation times in FoxP2 knockdown and control bids.

One possible explanation for accelerated AFP signal propagation times following FoxP2 KD is that diminished signaling through the D1R+ pathway unmasks or accentuates other pathways. One such pathway is the cortico-thalamocortical loop (HVC-RA-DLM-LMAN) that is also capable of driving LMAN activity (Goldberg and Fee, 2012; Wild, 1993; Vates et al., 1997). DLM neurons are strongly modulated by singing even in the absence of input from Area X (reversible inactivation or lesion) suggesting that this pathway is capable of driving LMAN. These findings raise the possibility that FoxP2 knockdown reduces the inhibition that Area X pallidal neurons exerts on DLM, therefore disinhibiting DLM neurons that in turn could increase the influence that RA neurons exert on LMAN activity (Goldberg and Fee, 2012). Since this alternate pathway involves gluatamergic synapses, it can lead to faster signal propagation times observed in the FoxP2 knockdown animals. Furthermore, disinhibition of DLM neurons can result in elevated firing rates and burst firing of LMAN observed in FoxP2 knockdown animals.

In order to test if the HVC-RA-DLM-LMAN pathway contributed to accelerated signal propagation times observed in the FoxP2 knockdown animals, I used *in vivo* intracellular sharp recordings of LMAN neurons while stimulating HVC and combining it with pharmacological manipulation of Area X (SKF application) in anaesthetized LV-ShFoxP2 and ShC injected birds that had received prior RA lesions (electrolytic lesion).

Preliminary findings (one neuron each from ShFoxP2 and ShC birds) show that RA lesions (~ 70%) had no effect on signal propagation times in both FoxP2 knockdown and control birds (Figure 25). In the LMAN neuron recorded from the ShC bird with RA lesion exhibited a transient increase in signal propagation times with SKF application, similar to the effects of SKF application in control animals with an intact RA (see section 4.1.2). Likewise, the AFP signal propagation times in the ShFoxP2 knockdown bird with RA lesion remained insensitive to application of SKF in a manner similar to their RA intact ShFoxP2 counterparts. Because these lesions were without any effect, but were also not quite large enough to encompass all of RA, these lines of experiments were not pursued further.

Conclusions:

Ultimately, while these experiments do not completely rule out the possibility that HVC-RA-DLM-LMAN pathway could play a role in the accelerated signal propagation times observed in the FoxP2 knockdown animals, we think this explanation is unlikely. It will be an important future direction to circuit mechanism that result in accelerated AFP signal propagation times in the FoxP2 knockdown animals (see section 5.4.3).

Methods

A day prior to the *in vivo* intracellular experiment, birds were anaesthetized with 2% isofluorane and a stainless steel post was mounted on the bird's skull with dental cement. RA was lesioned electrolytically by passing current through a 0.1 MΩ tungsten electrode (10 μA, 5 s duration, 6 times at each site). 3-4 sites were chosen to span most of RA. On the day of the experiment, the birds were anaesthetized with either 20% urethane injected into their pectoral muscles. The target nuclei (coordinates: LMAN- 5.3 mm rostral, 1.9 mm lateral, HVC – 0 mm caudal, 2. 4 mm lateral and Area X- 6.3 mm rostral, 1.6 mm lateral or 5.3 mm rostral and .5 mm lateral) were measured from the midsagittal sinus bifurcation and craniotomies (~200 μm by 200μm) were made over the target nuclei (LMAN, HVC and Area X). Sharp intracellular recordings of LMAN neurons were obtained while HVC was electrically stimulated using bipolar tungsten electrodes (0.1 MΩ, Microprobes) with the tips spaced ~300 μm. Biphasic stimulation for 400 μs was applied at a current strength of 40 μA. Synaptic latency and amplitude of postsynaptic potentials (PSPs) of LMAN responses to HVC stimulation were measured from median filtered traces using custom event detection software (Matlab, K.Hamaguchi). A glass pipette (tip diameter 15–20 μm) was attached to a tungsten electrode (0.5–1.0 MΩ, Microprobes). The glass pipette was filled with either 0.5 mM SKF 38393 hydrobromide (Tocris Bioscience) in .9% saline and ~30-60 nl was injected using a Picospritzer II (General Valve) in 50–150 ms pulses at 30 psi (Leblois et al., 2010). The tungsten electrode was used to record multiunit neural activity

in Area X and electrode placement was confirmed on the basis of robust responses in Area X to HVC stimulation. The glass pipette-tungsten electrode into Area X was lowered at a 25-30° to avoid passing through LMAN. Standardized two-tailed t-tests for independent samples were performed to test for statistical significance between the control and the knockdown groups. Nissl Staining was used to confirm RA leisons. A two-factor ANOVA with repeated measures on one factor was used to test for statistical significance in latency measurements followed by post hoc two-tailed t-tests for correlated samples.

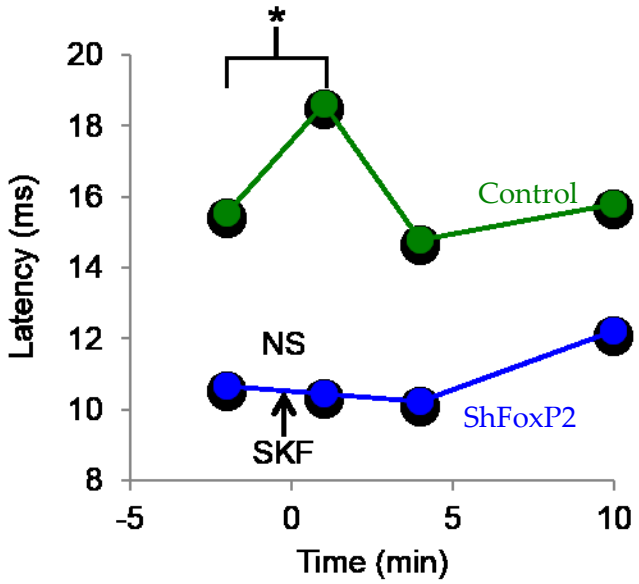

Figure 25: RA lesions have no effect on signal propagation times in both FoxP2 knockdown and control animals

In adult male zebra finch control with RA lesion (green, n=1 cell, 1 bird), the AFP signal propagation time transiently increases following the injection of D1R agonist (SKF) into Area X. In contrast, the AFP signal propagation time in adult finches previously injected with LV-ShFoxP2 and with RA lesions (blue, n = 1 cell, 1 birds) is unaffected by the SKF injection.

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Biography

My name is Malavika Murugan. I was born in Madras (now Chennai), India on the February 7th, 1986. I attended the Vellore Institute of Technology (Vellore, India) and obtained a Bachelor of Technology in Biotechnology in May 2007. I was part of a publication titled "Motor circuits are required to encode a sensory model for imitative learning". Since obtaining my bachelor's degree, I have been awarded the Ruth Broad Graduate Student fellowship.